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STUDIES ON THE PLASTICITY OF GLUTAMATE RECEPTORS AND ITS ROLE FOR NEURONAL CALCIUM HOMEOSTASIS

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Studies on the Plasticity of Glutamate Receptors and its Role for Neuronal Calcium Homeostasis
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Calcium is the most important and versatile signaling molecule in the brain. Neurons are equipped with a variety of tools to utilize this simple ion in diverse ways. From controlling the millisecond fast neurotransmitter release, to reshaping neuronal structure and function. A brief pulse of glutamate, released from a presynaptic neuron, activates the NMDA receptor and mGluR5 which triggers an increase in the cytosolic calcium concentration in the postsynaptic neuron. The NMDA receptor and mGluR5 are important mediators of synaptic plasticity - the ability of synapses to change their strength in response to changed patterns of neuronal activity. Because of the fundamental role calcium signaling has for numerous processes in the brain it is also highly sensitive to disturbances. A sustained high level of calcium can even cause neuronal death. We have in this work studied the plasticity of neuronal glutamate receptors and how novel accessory proteins can modulate their calcium mobilization.

In study I, we identified Norbin as a novel interacting partner to mGluR5. Norbin facilitated the cell surface expression of mGluR5 and positively modulated mGluR5 mediated calcium signaling. Studies in Norbin transgenic animals confirmed and demonstrated the relevance of this interaction. Mice lacking Norbin displayed decreased cell surface expression of mGluR5, impaired synaptic plasticity, and a behavioral phenotype that corresponds to hypofunctional mGluR5 activity.

In study II, using super-resolution imaging, we investigated the spatial relationship between Norbin, postsynaptic density protein 95 (PSD-95), mGluR5, and actin in dendritic spines. We found Norbin to have a high degree of colocalization with actin and a much lower degree with PSD-95. We also found that Norbin, as expected, have a high degree of colocalization with mGluR5 and the mGluR5-interacting protein Homer. Co-immunoprecipitation experiments confirmed our imaging results.

In study III, we identified p11 as another novel partner to mGluR5. Similar to Norbin, p11 promoted mGluR5 plasma membrane expression and modulated mGluR5 cell signaling. The known antidepressant effect of the mGluR5 specific antagonist MPEP was abolished in p11 knockout mice with a specific deletion of p11 in parvalbumin positive GABAergic interneurons.

In study IV, we investigated the spatial and functional relationship between the NMDA receptor and Na^+,K^+-ATPase in cultured hippocampal neurons. We found that sub-saturating concentrations of ouabain, a Na^+,K^+-ATPase ligand, attenuated NMDA receptor mediated calcium influx. Experiments performed in HEK293 cells indicated that the effects of ouabain was mediated by the neuronspecific Na^+,K^+-ATPase α3 isoform. Using single-molecule super-resolution microscopy dSTORM, we found a close spatial proximity between the NMDA receptor and the Na^+,K^+-ATPase in postsynaptic membranes.

LIST OF SCIENTIFIC PAPERS

I. Hong Wang, **Linda Westin**, Yi Nong, Shari Birnbaum, Jacob Bendor, Hjalmar Brismar, Eric Nestler, Anita Aperia, Marc Flajolet, Paul Greengard,

Norbin Is an Endogenous Regulator of Metabotropic Glutamate Receptor 5 Signaling.
Science, 2009, 326, 1554-1557.

II. **Linda Westin**, Matthias Reuss, Maria Lindskog, Anita Aperia, Hjalmar Brismar.

Nanoscopic spine localization of Norbin, an mGluR5 accessory protein.
BMC Neuroscience, 2014,15:45.

III. Ko-Woon Lee, **Linda Westin**, Jeongjin Kim, Jerry C. Chang, Yong-Seok Oh, Bushra Amreen, Jodi Gresack, Marc Flajolet, Daesoo Kim, Anita Aperia, Yong Kim, Paul Greengard.

Alteration by p11 of mGluR5 localization regulates depression-like behaviors.
Molecular Psychiatry, 2015, 20, 1546-1556

IV. **Linda Westin**, Evgeny Akkuratov, Minttu de Marothy, Alexandra Melnikova, Hans Blom, Maria Lindskog, Hjalmar Brismar, Anita Aperia.

Instantaneous down-regulation of NMDA receptor activity by nanomolar concentrations of ouabain.
Manuscript.

Additional papers not included in this thesis

- Hjalmar Brismar, Anita Aperia, **Linda Westin**, Jeffrey Moy, Mei Wang, Chistelle Guillermier, Collin Poczatek, Claude Lechene.

  Study of protein and RNA in dendritic spines using multi-isotope imaging mass spectrometry (MIMS).
  Surf Interface Anal, 2014, 1; 46(Suppl 1): 158–160.

- Hans Blom, Daniel Rönnlund, Lena Scott, **Linda Westin**, Jerker Widengren, Anita Aperia, Hjalmar Brismar.

  Spatial distribution of DARPP-32 in dendritic spines.
  PloS One, 2013, 8(9):e75155.

CONTENTS

LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ATP	Adenosine triphosphate
CaMKII	Calcium/calmodulin-dependent protein kinase II
CNS	Central nervous system
DAG	Diacylglycerol
D-APV	2-amino-5-phosphonopentanoic acid
DHPG	(RS)-3,5-dihydroxyphenylglycine
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FRAP	Fluorescence recovery after photobleaching
Fyn	Fyn member of the Src family of tyrosine kinases
GABA	γ-aminobutyric acid
GAD-65/67	Glutamate decarboxylase 65/67
GCaMP	Genetically encoded calcium indicator
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
IP_3	Inositol trisphosphate
KO	Knockout
LTD	Long-term depression
LTP	Long-term potentiation
MAP2	Microtubule-associated protein 2
mGluR	Metabotropic glutamate receptor
MPEP	2-metyl-6-(phenylethylnyl)pyridine
mRNA	Messenger ribonucleic acid
NCX	Na^+/Ca^{2+} exchanger
Na^+,K^+-ATPase α1	Na,K-ATPase alpha 1 subunit
Na^+,K^+-ATPase α3	Na,K-ATPase alpha 3 subunit
NMDA	N-methyl-D-aspartic acid
PALM	Photoactivated localization microscopy
PKC	Protein kinase C
PLC	Phospholipase C
PSD	Postsynaptic density
PSD-95	Postsynaptic density protein 95
SFK	Src family of tyrosine kinases
SIM	Structured illumination microscopy

STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
VGluT1	Vesicular glutamate transporter 1
WT	Wild-type

1 INTRODUCTION

1.1 NEURONAL CA^{2+}

Ca^{2+} is frequently referred to as the most universal signaling molecule. Virtually all cells are capable of responding to changes in cytosolic Ca^{2+}, although they carry varying molecular tools to gate, sense and respond. At rest the neuronal Ca^{2+} concentration is approximately 50-100 nM, which is up to 10.000 fold lower than the extracellular Ca^{2+} concentration and the concentration in the membrane-bound Ca^{2+}-stores such as the endoplasmic reticulum (ER), creating a considerable electrochemical gradient for Ca^{2+} across plasma and organelle membranes (Berridge et al, 2000; Grienberger & Konnerth, 2012). The intracellular Ca^{2+} concentration is defined by the complex interplay between factors that regulate Ca^{2+} influx and efflux as well as the exchange of Ca^{2+} between intracellular stores and the cytosol (Berridge et al., 2000). During high activity the Ca^{2+} concentration can temporarily rise to 1000 nM (Berridge et al., 2000; Grienberger & Konnerth, 2012).

Ca^{2+} regulates diverse neuronal processes ranging from milliseconds to minutes or hours. A transient rise through voltage-gated Ca^{2+} channels in presynaptic terminals leads to the exocytosis of neurotransmitter-containing vesicles. In dendritic spines, changes in cytosolic Ca^{2+} levels via activation of N-methyl-D-aspartic acid (NMDA) receptors or group 1 metabotropic glutamate receptors (mGluRs) may generate long-lasting changes in synaptic strength by regulating α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor trafficking and local protein synthesis (Grienberger & Konnerth, 2012; Lüsher & Malenka, 2012; Huber et al., 2000; Holbro et al., 2009).

Some of the actors that regulate neuronal Ca^{2+} signaling are Ca^{2+}-permeable ion channels located in the plasma membrane, such as voltage-gated Ca^{2+} channels and ionotropic glutamate receptors. These ion channels regulate Ca^{2+} influx from the extracellular space in response to changes in membrane potential and/or glutamate release from presynaptic cells. The plasma membrane Ca^{2+} ATPase (PMCA) and the sodium-calcium exchanger (NCX) located in the plasma membrane counteract Ca^{2+} influx and extrude Ca^{2+} from the cytosol to the extracellular milieu using ATP or the sodium gradient as driving force. In addition, Ca^{2+} channels located in the membrane of the ER, the IP_3 receptor and ryanodine receptors (RyR), may release Ca^{2+} upon binding IP_3 produced by activation of mGluRs and/or via Ca^{2+} induced Ca^{2+} release respectively (CICR). Ca^{2+} can be pumped from the cytosol into the sarco/endoplasmic reticulum by the Ca^{2+}-ATPase (SERCA) (Berridge, 1998; Berridge et al., 2000; Grienberger & Konnerth, 2012).

Changes in the cytosolic Ca^{2+} concentration can come in various patterns ranging from a brief Ca^{2+} pulse to sustained increases or persistent oscillations depending on the means of activation. The downstream effects of Ca^{2+} is determined by the activation of multiple Ca^{2+} binding proteins located in the cell such as CaMKII, calcineurin and calmodulin. Both the location, amplitude and temporal patterning of Ca^{2+} can encode for a variety of cellular functions by activating distinct Ca^{2+} binding proteins (Berridge et al., 2000; Smedler & Uhlén, 2014). Sustained high levels of cytosolic Ca^{2+} can however be toxic and trigger mitochondria dependent cell death (Lipton & Rosenberg, 1994; Simon et al., 1984; Lemasters et al., 2009).

1.2 SYNAPSES

The nerve cell, or neuron, is the main basic signaling unit of the brain. Neurons are highly polarized, electrically excitable cells that receive, process and transmit electrochemical information. Neurons may form networks by communicating at specialized cell-cell junctions called synapses. The vast number of excitatory synapses between presynaptic axon terminals and postsynaptic dendrites reside on small dendritic protrusions called dendritic spines which are highly specialized compartments that both receive, process and partly confine synaptic input.

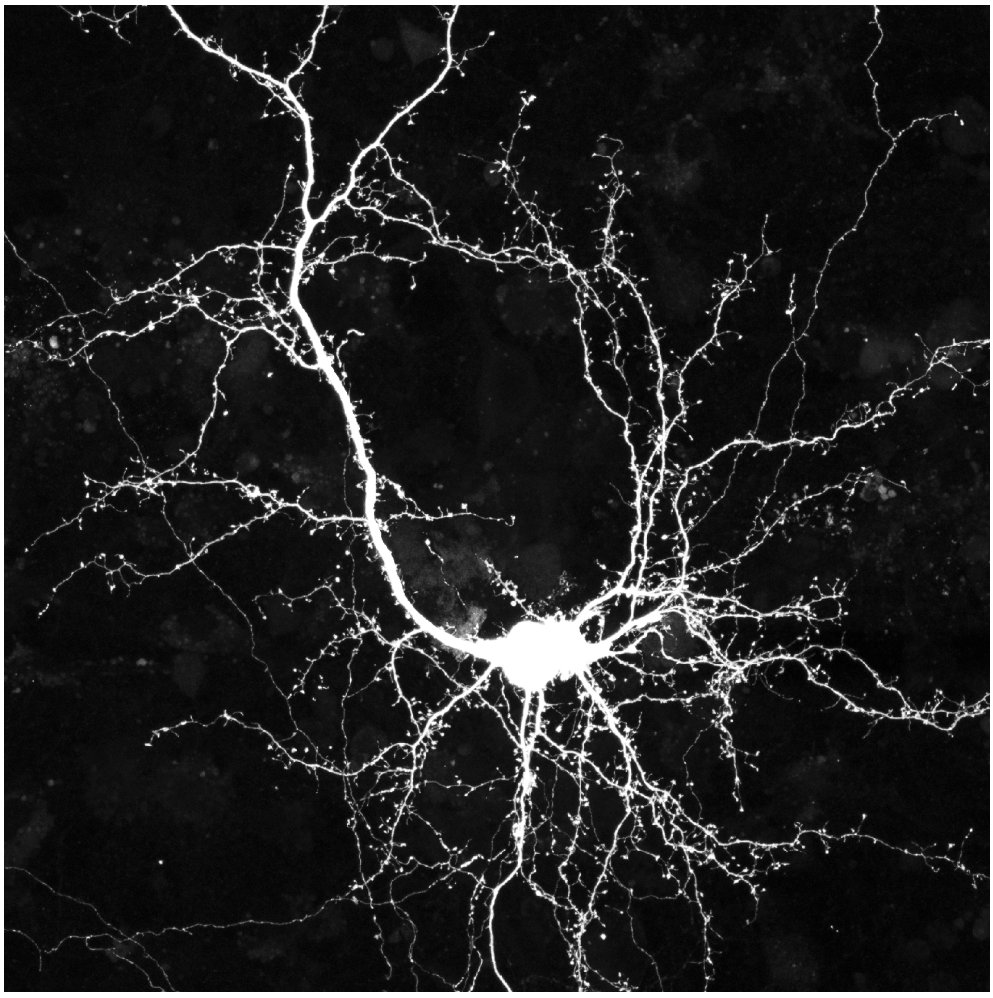

Figure 1.1 A hippocampal neuron with long extending dendrites each containing numerous dendritic spines.

1.3 GLUTAMATE

Glutamate, a single amino acid, is the principal excitatory neurotransmitter utilized by the mammalian brain. Glutamate can be synthesized de novo via Krebs cycle or from glutamine. It is stored at high concentrations in presynaptic vesicles and released into the synaptic cleft via exocytosis in a Ca^{2+}-dependent manner. Glutamate diffuses across the synaptic cleft and binds to glutamate receptors generally located dendritic spines (Lüsher & Malenka, 2012). Glutamate receptors on the receiving postsynaptic membrane are either ligand-gated ionotropic (ion-channels) or metabotropic (signal-transducers). Glutamate modulates diverse neuronal functions such as synaptic plasticity, gene expression and cell survival by interacting with ionotropic and metabotropic glutamate receptors.

1.4 IONOTROPIC RECEPTORS

There are three types of ionotropic glutamate receptors expressed in the mammalian brain AMPA, NMDA and kainate receptors which are named after their preferred synthetic agonists (Monaghan et al., 1989). These receptors constitute ion-channels that are permeable to Na^+ and Ca^{2+} and slightly to K^+. Once activated they allow for a strong net influx of positive ions (an inward current) which causes a postsynaptic membrane depolarization. AMPA receptors respond rapidly to glutamate and have fast kinetics, are generally highly permeable to Na^+ and mediate the fast excitatory input within a few milliseconds following glutamate release (Lüsher & Malenka, 2012). Once activated they are rapidly desensitized.

NMDA receptors are believed to exist as heterotetrameric complexes primarily comprised of two essential NR1 subunits and two NR2 subunits (Paoletti et al., 2013). There are eight known NR1 splice variants. The four known NR2 subunits (NR2A, NR2B, NR2C and NR2D) are encoded by four different genes. NR2A and NR2B are the predominant subunits expressed in human and rodent forebrain. The heterotetrameric subunit composition defines the kinetics of the receptor (Paoletti et al., 2013). NMDA receptors are highly permeable to Ca^{2+} and Na^+ (Lüsher & Malenka, 2012). An important feature of the NMDA receptor is that it is blocked by a Mg^{2+} ion obstructing the ion channel pore under resting conditions. The Mg^{2+} is expelled after a sufficient membrane depolarization following e.g. robust AMPA receptor activation. Since the NMDA receptor, due to the Mg^{2+} block, requires both the pre- and postsynaptic neuron to be activated it is often referred to as a coincidence detector (Lüsher & Malenka, 2012). In addition, the NMDA receptor requires the binding of both glutamate, and the co-agonists glycine or D-serine (Meldrum, 2000).

1.5 METABOTROPIC GLUTAMATE RECEPTORS

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors (GPCRs) that are linked to G-proteins located in the interior of the cell. Ligand binding to GPCRs result in conformational changes which activates G-proteins resulting in various downstream signaling cascades that ultimately modulate enzymes, ion channels and transcription factors (Niswender & Conn, 2010).

mGluRs are part of the class C GPCRs and contain seven transmembrane domains, a large ligand binding N-terminus (Venus flytrap domain), and an intracellular C-terminus which is regulated by protein-protein interaction and phosphorylation. There are eight identified mGluR subtypes encoded by eight genes which can be divided into three groups (group I, II and III) based on their sequence homology, G-protein coupling and ligand selectivity. Group 1 includes mGluR1 and mGluR5 which are $G_{\alpha q}$ coupled (Niswender & Conn, 2010).

Glutamate binding to group 1 mGluRs activates phospholipase C (PLC). PLC triggers the formation of diacylglycerol which activates protein kinase C (PKC), and generates inositol-1,4,5-trisphosphate (IP_3). IP_3 binds to the IP_3 receptor (IP_3R) located in the ER, which leads to the release of Ca^{2+} from ER. mGluR5 activation triggers the generation of cytosolic Ca^{2+} oscillations (Kawabata et al. 1996; Conn and Pin, 1997). The signaling generated by group 1 mGluR has been shown to activate several pathways that are thought to be important in synaptic plasticity such as extracellular signal-regulated kinase (ERK), mammalian target of rapamycin (MTOR) (Niswender & Conn, 2010).

While mGluRs belonging to groups II and III are primarily located presynaptically, group 1 mGluRs are preferentially located on postsynaptic membranes (Niswender & Conn, 2010). mGluR5 is widely distributed in the brain with a prominent expression in e.g. hippocampus and striatum (Romano et al., 1995). mGluR5 can be found with high expression in both principal and non-principal cells in hippocampus (Luján et al., 1996). mGluR5 can exist as at least two splice variants, mGluR5a and mGluR5b with developmentally complementary profiles (Minakami et al., 1995).

The oscillations triggered by mGluR5 activation can be seen in heterologous expression systems but reports on their presence in supposedly homologous cells vary. Cultured astrocytes which only express mGluR5, show PKC-sensitive Ca^{2+} oscillations after agonist application (Nakahara et al, 1997). Developing cortical neurons display spontaneous mGluR5-triggered Ca^{2+} oscillations (Flint et al., 1999), while reports on the occurrence of mGluR5 associated oscillations in cultured hippocampal neurons vary (Rae et al., 2000, Woodhall et al., 1999).

1.6 MGLUR5 FUNCTION

The Ca^{2+} and PKC signaling cascades generated by mGluR5 activation can trigger a variety of cellular responses that are important during embryonic development and in regulating the synaptic strength between neurons in the adult brain. A large interest for mGluR5 has in part come about from observations that it can modulate NMDA receptor function in widespread CNS regions. Reportedly, mGluR5 facilitate NMDA receptor function in striatum, cortex, and hippocampus (Pisani et al., 2001; Attucci et al., 2001; Doherty et al., 2000; Mannaioni et al., 2001). mGluR5 knockout mice display reduced NMDA dependent excitatory postsynaptic currents in hippocampus, impaired spatial learning and attenuation of NMDA dependent synaptic plasticity (Lu et al., 1997; Jia et al., 1998; Bortolotto et al., 2005). The regulation has been proposed to depend on both a physical coupling via scaffolding proteins in the postsynaptic density as well as changes in PKC activity.

1.7 DENDRITIC SPINES

The vast number of glutamatergic synapses in the mammalian brain occur at dendritic spines. Although glutamate receptors are the primary target of excitatory synaptic activity, they are not isolated but found in structural and functional assemblies with a large number of other postsynaptic molecules. Dendritic spines are typically 0.5-2 μm long, with bulbous heads and thin necks connecting the spine head to the parent dendrite (Sheng & Hoogenraad, 2007). A single neuron can host thousands to tens of thousands of spines. Live imaging both in vitro and in vivo, has shown that spines are highly dynamic structures, even in adulthood (Holtmaat et al., 2005). Changes in spine morphology typically correlate with synaptic strength and AMPA receptor abundance. Spines are structurally highly organized, despite their plastic capacity. The core components of dendritic spines are: an electron dense region adjacent to the presynaptic site of glutamate release called the postsynaptic density (PSD), the actin cytoskeleton, and organelles such as the smooth endoplasmic reticulum, mitochondria and endosomes (Hotulainen & Hoogenraad, 2010).

The PSD is built of hundreds of specific proteins, has a laminar organization and serves as to anchor glutamate receptors with their signaling molecules at the active site (Sheng &

Hoogenraad, 2007; Valtschanoff & Weinberg, 2001, Dani et al., 2010). AMPA and NMDA receptors are momentarily stabilized by coupling to PSD proteins located just beneath the plasma membrane such as multimeric complexes of postsynaptic-density 95 (PSD-95) (Ehlers et al., 2007; Nair et al., 2013). mGluR5 accumulates in the periphery of the active site (perisynaptically) and is stabilized in the spine by directly interacting with members of the Homer family which act as scaffolding molecules (Lujan et al., 1996; Brakeman et al., 1997; Sergé et al., 2002). Homer proteins form network structures just beneath the PSD-95 dense regions in the axiodendritic axis (Valtschanoff & Weinberg, 2001, Dani et al., 2010). The mGluR5 and NMDA receptors are coupled via PSD protein chains such as PSD-95, SHANK, GKAP and Homers (Tu et al., 1999; Naisbitt et al., 1999) which enables a direct crosstalk between the receptors (Perroy et al., 2008). CaMKII is one of the most abundant components of the PSD (Sheng & Hoogenraad, 2007), suggesting the imperative role of Ca^{2+}-dependent signaling cascades in synaptic activity. The actin cytoskeleton is highly enriched in dendritic spines and plays an important role in the dynamic reshaping of the spine structure, PSD composition and glutamate receptor trafficking during synaptic activity (Hotulainen & Hoogenraad, 2010).

The interaction between glutamate receptors and associated proteins that affect receptor trafficking are important for synaptic function. mGluR5 localization and signaling is tightly regulated by its interaction with Homer in the spine. Homer confines mGluR5 within clusters in the spine and counteracts the dispersion caused by receptor activation (Sergé et al., 2002). Disruptions of mGluR5-Homer interactions can cause abnormal behavior seen in Homer knockout mice (Ronesi et al., 2012). Both Homer and Shank proteins, which cross-links mGluR5 to the NMDA receptor via PSD-95, have been shown to influence mGluR5 function.

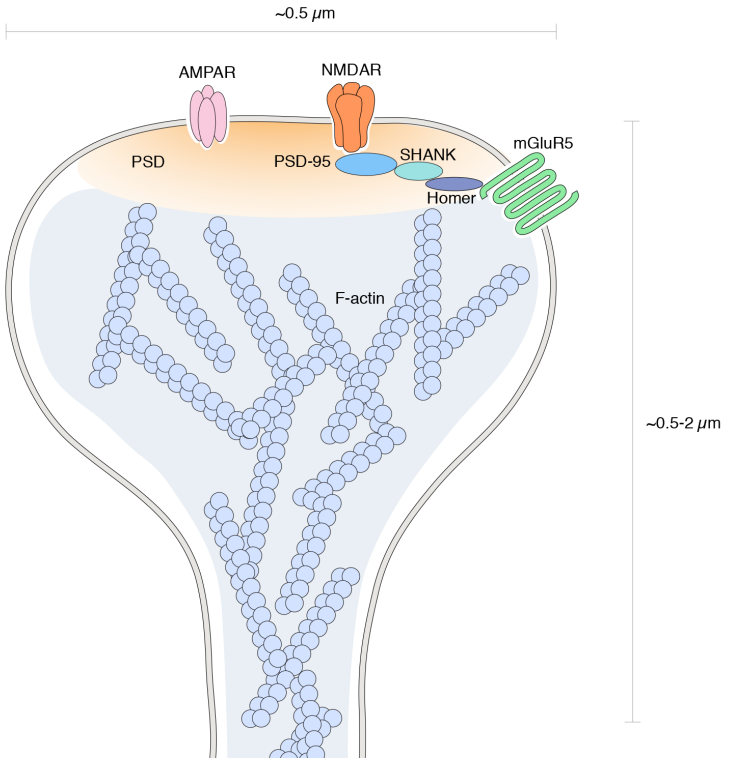

Figure 1.2 An illustration of the dendritic spine containing the PSD, glutamate receptors and scaffolding molecules.

1.8 SYNAPTIC PLASTICITY

Long-lasting experience dependent changes in the synaptic strength between neurons are thought to be the cellular substrate underlying learning and memory and important to many aspects of cognitive function. Two well-studied forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD). LTP correlates to a long-lasting increase in synaptic strength and enlarged spine heads, while LTD correlates to a long-lasting decrease in synaptic strength and decreased spine heads. The most studied types of LTP and LTD typically require the activation of NMDA receptors and group 1 mGluRs.

CA1 hippocampal mGluR5-dependent LTD can be induced by low frequency stimulation or short-term bath application of (RS)-3,5-dihydroxyphenylglycine (DHPG), a group 1 mGluR specific agonist (Huber et al., 2000). This form of long-term plasticity is abolished using cycloheximide or anisomycin which suggest that mGluR5 activation regulate protein synthesis, possibly at a local protein synthesis machinery which can be found at the base of the spine (Huber et al., 2000; Sutton & Schuman, 2006). mGluR5 KO mice and mice treated with MPEP show a deficiency in CA1 LTP, possibly by disrupting the protein synthesis dependent late phases of LTP (Lu et al, 1997; Jia et al., 1998; Bortolotto et al., 2005). LTP and LTD fundamentally involves the regulation of AMPA receptor abundance at the active site, which correlates to the synaptic strength.

Generally, the level of glutamate triggered Ca^{2+} increases are thought to define the physiological readout, such that a small prolonged increase in Ca^{2+} will induce LTD, while a large increase will induce LTP (Lüsher & Malenka, 2012). The cellular readout is defined by which Ca^{2+} binding proteins are activated by the change in Ca^{2+}. Typically, CaMKII activation is required for LTP, while calcineurin is required for LTD. However, the role of Ca^{2+} in synaptic plasticity has been challenged recently, and some forms of plastic phenomena have been shown to be Ca^{2+} independent (Nabavi et al., 2013). Nevertheless, numerous studies have implicated the necessity of Ca^{2+} flux to regulate postsynaptic trafficking of AMPA receptors (Lüsher & Malenka, 2012).

1.9 GLUTAMATE RECEPTORS IN DISEASE

Balanced glutamatergic activity is critical to avoid diverse neuropathological states. Both hypo- and hyperfunctional glutamatergic activity can lead to severe cognitive deficits. Fragile X syndrome is the most common inherited form of mental retardation and is caused by a deficit of the fragile X mental retardation protein (FMRP) which negatively regulates dendritic mGluR5 mediated protein synthesis (Bear et al., 2004). The resulting exaggerated mGluR5-triggered protein synthesis seen in the Fmr1 KO mouse which lack FMRP, leads to increased LTD as well as increased density of long filopodia-like spines, cognitive deficits and ultimately severe mental disability (Bear et al., 2004). mGluR5 antagonists or negative allosteric modulators of genetic deletion can reverse several of the phenotype features in mouse models of fragile X syndrome (Krueger & Bear, 2011), suggesting a potent role for mGluR5 as a pharmacological target for neuropathological disease related to aberrant synaptic plasticity, even in adolescence or adulthood.

In contrast, hypofunction of mGluR5 is implicated in schizophrenia (Pietraszek et al., 2006; Conn et al., 2009). The underlying molecular and cellular mechanisms of this complex

disease are still elusive and appear not be caused by any single gene. Even so, studies showing that NMDA receptor antagonists phencyclidine (PCP) and ketamine could induce schizophrenia-like symptoms in healthy humans, has led to the NMDA receptor hypofunction hypothesis of schizophrenia (Coyle, 2012). Subsequent observations demonstrated that ligands that stimulate NMDA receptor activity at the glycine allosteric site, could improve cognition in schizophrenic patients (Coyle, 2012). These observations suggested a role of the glutamatergic system in schizophrenia. mGluR5 knockout mice and mice treated with mGluR5 specific antagonists display robust schizophrenia related behavior observed across species including an impairment in pre-pulse inhibition (PPI) and NMDA receptor antagonist induced hyperlocomotor activity (Pietraszek et al, 2006).

Current treatment for major depression disorder (MDD) is mainly based on targeting the serotonergic system using selective serotonin reuptake inhibitors (SSRIs). However, there is delay in the onset of pharmaceutic efficacy as well as incomplete efficacy, and side effects of the treatment (Svenningsson et al., 2013). A growing number of studies implicate a role of glutamate receptor related dysfunction and clinical and preclinical studies suggest that pharmacological treatment targeting the NMDA receptor and mGluRs, can alleviate symptoms of depression. In particular NMDA receptor and mGluR5 antagonists have shown antidepressant efficacy in mouse models of depression (Chaki et al., 2013).

Persistent activation of glutamate receptors during excessive glutamate release can cause an excitotoxic increase in cytosolic Ca^{2+} levels. NMDA receptor overactivation and subsequent dysregulated Ca^{2+} homeostasis is thought to be the underlying cause of neuronal death following brain ischemia and traumatic brain injury (Lipton & Rosenberg, 1994).

Considering that the NMDA receptor and mGluR5 are such fundamental regulators of synaptic efficacy and normal brain function, knowledge about their endogenous regulators are highly important.

1.10 INTERACTING PROTEINS

This section provides a brief description of the proteins that we found as regulators of NMDA receptor and mGluR5 function.

1.10.0 Norbin

Norbin (neurite outgrowth related protein in rat brain), also named neurochondrin, is a neuronspecific cytosolic protein that was initially identified in an assay screening for genes upregulated after tetraethylammonium (TEA) induced LTP in hippocampus (Shinozaki et al., 1997). Norbin is expressed in the brain, peripheral nervous system, chondral and bone tissue. A prenatal deletion of Norbin in mice leads to early embryonic death at embryonic day E3.5-6.5 (Mochizuki et al., 2003). Norbin can be detected in mouse CNS by in situ hybridization at E10 and onwards and increases in protein expression up to postnatal day 21 (Wang et al., 2010). Ectopic expression of Norbin in Neuro2A induced neurite outgrowth which suggest that Norbin might play a role in dendritic outgrowth (Shinozaki et al., 1997). Norbin has a somatodendritic distribution and is widely distributed in the adult mouse brain but is particularly highly expressed in the amygdala, hippocampus, and cerebellum and has a moderate expression in cortex and striatum (Wang et al., 2010). Norbin displays a strong

expression in hippocampal pyramidal neurons and granule cells in dentate gyrus and in cortical layers II and IV. Although Norbin is primarily cytosolic, a fraction is triton insoluble, indicating that it might also associate with membrane and cytoskeletal factors (Shinozaki et al., 1999). Norbin has, since its identification, been shown to interact with a few receptors such MHCR1 and Sema4C, and other molecules such as phosphatidic acid and Dia1 (Wang et al, 2010). Mice with a postnatal disruption of the Norbin gene display impaired spatial learning and memory and are prone to epileptic seizures in adulthood (Dateki et al., 2005).

1.10.1 P11

P11 is a small cytosolic protein and is part of the S100 EF-hand protein family. Unlike other members of the S100, p11 is Ca^{2+} insensitive due to mutations in the Ca^{2+} binding sites of the EF-hands. P11 form heterotetrameric complexes with Annexin A2 (AnxA2) which binds to and reorganizes membrane lipids and the actin cytoskeleton. p11 is widely expressed in regions implicated in depression such as nucleus accumbens, cerebral cortex and hippocampus. A particularly strong expression is found in cortical layer 5 pyramidal neurons, hippocampal calretinin mossy cells and parvalbumin expressing inhibitory neurons, and cholinergic neurons in nucleus accumbens. P11 has been found to interact with several members of the 5HT family of serotonin receptors and regulate receptor trafficking, increase receptor membrane expression and modify serotonin mediated cell signaling. The role of p11 in depression-like states has been studied in recent years. Constitutive P11 KO mice display depression-like behavior and the anti-depressant effect of SSRIs and other monoaminergic anti-depressants are in part mediated by p11. P11 mRNA is decreased in patients suffering from MDD and suicide victims while monoaminergic anti-depressant treatment increases p11 mRNA, implicating an important role for p11 in depression (Svenningsson et al., 2013).

1.10.2 Na^+,K^+-ATPase

Last but not least, the vital Na^+,K^+-ATPase, which can be found in all eucaryotic cells. The electrogenic transport performed by the Na^+,K^+-ATPase as it pumps three Na^+ out of, and two K^+ in to the cell at the expense of one adenosine triphosphate (ATP) molecule, provides all cells with a steep electrochemical gradient across the plasma membrane, and a negative membrane potential (Skou, 1957). The resulting gradient serves as a driving force for numerous crucial cell biological processes. The Na^+,K^+-ATPase is a heterotrimeric complex assembled from three distinct subunits, the catalytic ion-pumping α subunit which contains the Na^+, K^+, and ATP binding sites, the β subunit which is responsible for trafficking the α subunit to the membrane, and the regulatory γ subunit. The α subunit includes ten transmembrane domains, intacellular N- and C-termini, and two large and two small intracellular loops. There are four isoforms of the α subunit present across tissue: the α1 which is commonly referred to as essential and ubiquitosly expressed in all cells, the α2 which is mainly found in skeletal muscle cells and astrocytes in the adult brain, the α3 which is neuronspecific and the α4 which as to date has only been found in testes. A prenatal disruption of either of α1, α2 or α3 leads to embryonic or early postnatal death.

The different α isoforms have different affinities to Na^+, K^+, ATP, and the Na^+,K^+-ATPase specific ligand ouabain. In neurons, which express only the α1 and α3 isoform, these differences indicate that the Na^+,K^+-ATPase α isoforms could be activated in response to

different levels of neuronal activity. The α1 isoform has a higher affinity to Na^+ than the α3 isoform. The Na^+,K^+-ATPase α3 is responsible for sodium clearance only at very high neuronal activity, where Na^+ needs to be rapidly cleared (Azarias et al., 2013). In contrast, the highly specific Na^+,K^+-ATPase ligand ouabain, has a thousand fold higher affinity to Na^+,K^+-ATPase α3 than α1. At saturating concentrations, ouabain inhibits the pump which leads to membrane depolarization.

The Na^+,K^+-ATPase was early on described as a housekeeping enzyme and this has likely contributed to it being understudied, despite its importance. Particularly in the brain. A search in the pubmed database 2016-11-05 generated 10 fold less hits for the search term "*Na,K-ATPase neuron*" than "*NMDA neuron*". In spite of the fact that the identification of the Na^+,K^+-ATPase was done decades prior to that of the NMDA receptor. This should demonstrate the need for an increase in neuroscientific investigations concerning the role of this enzyme in neuronal function. Recent studies have however described novel roles of the Na^+,K^+-ATPase in multiple tissues and that the Na^+,K^+-ATPase can act as a signal transducer (reviewed in Aperia et al., 2016). In addition, single amino acid mutations of the Na^+,K^+-ATPase α3 have been shown to cause the rare diseases rapid-onset dystonia parkinsonism (RDP) and alternating hemiplegia of childhood (AHC). Two independent studies published 2015 show that α-synuclein and amyloid β assemblies bind to the identical extracellular amino acid of the Na^+,K^+-ATPase α3 which cause neurodegeneration by impairing the Na^+ gradient and disrupting Ca^{2+} homeostasis implicating a role for the Na^+,K^+-ATPase α3 in Parkinson's and Alzheimer's disease (Shrivastava et al., 2015; Ohnishi et al., 2015).

2 AIMS

The overall aim of this thesis was to investigate novel protein-protein interactions that may result in modulation of glutamate receptor function and their role in neurophysiology from an imaging based perspective.

Study I

Investigate the role of the neuronspecific protein Norbin/neurochondrin in regulating mGluR5 function.

Study II

To study the subcellular localization of Norbin in hippocampal neurons using novel super-resolution microscopy techniques.

Study III

To investigate the functional role of the interaction between p11 and mGluR5.

Study IV

To study the functional and spatial relationship between the Na^+,K^+-ATPase and the NMDA receptor in hippocampal neurons.

3 METHODOLOGY

This thesis includes two studies which were done in collaboration with the group of Paul Greengard at Rockefeller University, and two studies performed at the Karolinska Institute. The collaborative studies utilized a wide array of techniques spanning from identification and verification of protein-protein interactions to transgenic animal design, electrophysiology and behavioral tests. The work that I have performed was anchored in experimental imaging of labeled proteins and ion fluxes and therefore I will focus on the imaging based methods in this section rather than summarize the entirety of methods included in all four studies. I refer to the methodological section and supplementary material in each paper for a complete coverage of all experimental procedures included in the presented studies.

3.1 CELL CULTURE

In this work mainly two types of cell cultures have been used. For basic mechanistic studies which address functional aspects of protein-protein interaction, a simple cell line was used. We used HEK293 cells derived from human embryonic kidney cells which are commonly used to study basic features in protein function. The strength in using cell lines as model systems for key questions remain within their primitive nature. First of all, they are generally easy to handle and express exogenous plasmid DNA well. More importantly though, is the opportunity to isolate proteins such as receptors from their normally complex molecular environments and introduce them in simpler membranes where fewer endogenous regulations occur in order to get a clearer understanding of the direct actions in e.g. protein-protein interactions. In the Ca^{2+} studies in this thesis the majority of results were obtained from such cell lines. This was particularly important in study I, III and IV when we also studied the effect of interrupting protein-protein interactions in receptor function of mutated receptors unable to bind to their endogenous regulators. Their validity as a model to neuron specific protein-protein interactions should be addressed however, and if possible experiments should be validated in more relevant systems.

In study II and IV mainly primary cultures derived from Sprague Dawley rat embryos (E18-19) were used. Hippocampi were isolated, dissociated, seeded on coated glass coverslips and maintained for 3-4 weeks before experiments. After a few days in culture the dissociated cells start to form neurites to establish network activity. After 3 weeks in culture, the cells display extensive branching, are abundant in synapses with distinct pre- and postsynaptic structures (Fig. 3.1), express a plethora of neuronal proteins with expected localizations and show robust spontaneous Ca^{2+} activity, ranging from confined postsynaptic spine Ca^{2+} transients to synchronized network Ca^{2+} activity. Subpopulations of neurons stain positive for GAD-65/67 (Glutamate decarboxylase 65/67) or VGluT1 (Vesicular glutamate transporter 1), marking the presence of both inhibitory and excitatory neurons respectively. Astrocytes, recognized by a glial fibrillary acidic protein (GFAP), present in the culture also display branching and local Ca^{2+} activity. Even complex biological phenomena such as synaptic plasticity can be induced in primary hippocampal cultures. The clear drawback in studying primary cultures is the complete lack of original cellular organization and afferent input specificity. For hippocampal cultures this is a particularly relevant consideration, seeing to its highly precise cellular configuration and the activity dependent regulation of circuit function. However, the

questions addressed in this thesis have focused on molecular events in single cells and not network function. The key reason for using primary neuronal cultures have been the exceptional advantage in studying a monolayer of sparsely distributed cells using light microscopy to discern cellular and subcellular structures and events even at the molecular scale. Scattering of light in tissue in combination with a high labeling density can generally hamper the extraction of detailed information.

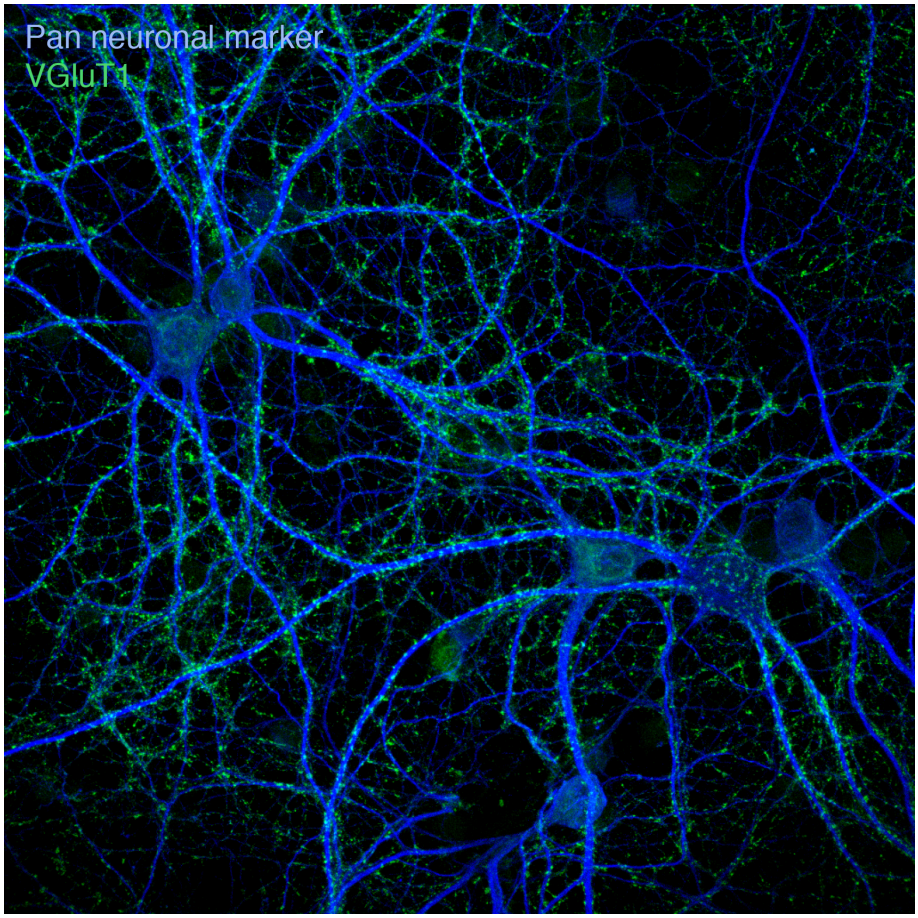

Figure 3.1 Cultured hippocampal neurons antibodylabeled for Pan neuronal marker and Vesicular glutamate transporter 1 (VGluT1).

3.2 IMMUNOCYTOCHEMISTRY AND TRANSFECTION

Essentially there are two classical ways two study protein distribution in cells. The first one is based on antibody labeling strategies in fixated tissue. The other is by expressing the protein of interest fused to a fluorescent protein which also enables the study of protein dynamics in living cells. We have used both these labeling strategies extensively in all projects and on occasion compared the distribution of endogenous and exogenous expression.

3.3 CALCIUM IMAGING

In three of the studies presented (I, III, IV), Ca^{2+} imaging was utilized as a tool to address questions on the physiological relevance of direct protein-protein interaction and the role of glutamate interacting proteins for receptor function. Ca^{2+} imaging was done both in cell lines and neuronal cultures using traditional Ca^{2+} sensitive dyes as well as recently developed genetic sensors.

Ca^{2+} imaging has been used extensively in biological sciences since the breakthrough in the development of fluorescent Ca^{2+} sensitive sensors in work led by Roger Tsien in the 1980s, of which many are still in broad use. The Ca^{2+} sensors used most frequently today can be defined as chemical Ca^{2+} indicators or genetically encoded Ca^{2+} indicators. The chemical (synthetic) Ca^{2+} sensors are based upon the use of Ca^{2+}-selective chelating chemicals such as ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid or 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, known as EGTA and BAPTA respectively. These chelators are particularly well suited in the engineering of fluorescent Ca^{2+} sensors due to their high selectivity (>10^5) to bind to Ca^{2+} compared to other divalent cations e.g. magnesium. This is an important feature, particularly in the study of cytosolic Ca^{2+} levels where the Ca^{2+} concentration is several orders of magnitude lower than magnesium. The use of BAPTA and EGTA in live cell Ca^{2+} imaging comes with the hybridization of these chelators with fluorescent chromophores. Broadly described, when Ca^{2+} binds the indicator undergoes a conformational change which changes the fluorescent properties of the chromophore.

Generally, the choice of Ca^{2+} sensor should be carefully considered, although several of them work for a wide range of biological questions and systems. Parameters to consider in choosing the appropriate dye are Ca^{2+} affinity (K_d i.e. the concentration of Ca^{2+} at which half of the indicator molecules are bound to Ca^{2+}) to ensure both sufficient sensitivity and avoidance of saturation, spectral properties, brightness and kinetics. Usually the loading conditions needs optimization depending on cell type and question. A nice feature with the chemical Ca^{2+} indicators is that they can be used in acetoxymethyl (AM) ester form. This enables the otherwise hydrophilic chemical to cross the plasma membrane. Estearases inside the cells then cleaves the AM groups which results in trapping of the dye. The AM ester form of bulk labeling synthetic Ca^{2+} dyes is a good alternative when studying a homogenous cell population.

In study I and III, chemical Ca^{2+} indicators Fura-2 developed in Roger Tsiens lab (Grynkiewicz et al., 1985) or Oregon Green 488 Bapta-1 were used. Fura-2 (K_d ~ 145 nM) is a ratiometric Ca^{2+} dye which shifts its peak absorbance from 380 nm in a Ca^{2+} free state, to 340 nm in a Ca^{2+} bound state. Its peak emission is around 510 nm. The ratio of the emission at 510 nm after separate excitation at 340 nm and 380 nm allows for quantitative measurements of the Ca^{2+} concentration, and a complete cancellation of contributions in fluorescence that usually depend on dye concentration and leakage, cell thickness, bleaching and focus drift. Oregon Green 488 Bapta-1 has a slightly higher K_d of ~ 170 nM, and a single excitation and emission peak. In the work presented here, Fura-2 was used when HEK293 cells were also expressing recombinant GFP (Norbin-GFP and p11-GFP). Oregon green 488 Bapta-1 was used when there was no need for a simultaneous detection of GFP.

Although the chemical dyes can be used to answer a variety of questions they come with a few drawbacks. First of all, Fura-2 is excited using ultraviolet illumination which can cause phototoxicity via the generation of reactive oxygen species (ROS). It is therefore essential to reduce the illumination intensity and duration. Secondly, the dye molecules tend to sequester in compartments within the cytosol. Although the loading procedure we used here were optimized with regard to loading duration, temperature and dye concentration, both

Oregon green 488 Bapta-1 and Fura-2 had a tendency to have a higher dye concentration within the nucleus.

An alternative approach that has gained interest in recent years are genetically encoded Ca^{2+} indicators (GECIs). These genetic sensors are broadly based on the use of fluorescent proteins containing a Ca^{2+}-binding domain such as the Ca^{2+} binding protein calmodulin. Of the many existing GECIs, the GCaMPs have proven to be highly useful in *in-vivo* imaging of neuronal activity. The GECIs have improved imaging conditions in multiple ways: the invasive procedure of dye loading is redundant, the GECIs can be made cell type or organelle specific by combining them with specific promoters and they can yield a high signal-to-noise (SNR) ratio. In study IV we used GCaMP6f (Chen et al., 2013) for imaging NMDA receptor-evoked Ca^{2+} influx in primary cultures and HEK293 cells. GCaMP6f has both a lower affinity (K_d ~375 nM) and faster kinetics than previous GCaMP versions making it optimal to study strong glutamate receptor stimulated Ca^{2+} influx. An inherent problem with Ca^{2+} dyes are the fact that they buffer Ca^{2+}, and therefore interfere with the cellular Ca^{2+} signaling much like endogenous Ca^{2+} binding proteins. Therefore, dyes that have fast on-off kinetics as well as a high SNR are highly attractive.

In study IV, when imaging Ca^{2+} activity in neurons, we started by comparing the bulk loading of Oregon green 488 BAPTA-1 with a transient transfection of GCaMP6f. Although Oregon green did report spontaneous Ca^{2+} activity throughout the neuronal structures, the activity continuously faded until neurons became completely quiet. The astrocytes in contrast displayed a significant rise in Ca^{2+} within 10-15 min. Decreasing dye concentration and illumination levels did not significantly improve these adverse effects during recordings. Outside the field of view, the neurons were still highly active, while astrocytes had stable baseline activity. Thus, it appeared as if the illumination, in combination with the dye, induced toxic events. In contrast, neurons transfected with GCaMP6f displayed distinct synaptic as well as network activity during extended imaging sessions, with low background fluorescence. Stimulation with NMDA or glutamate gave a large change in GCaMP6 fluorescence. We therefore utilized GCaMP6 as sensor in study IV (Fig. 3.2). Considering that these sensors are buffering Ca^{2+} to some extent, although with fast kinetics, we typically performed experiments 24-48 hours after transfection to avoid long-term effects of unphysiological Ca^{2+} buffering.

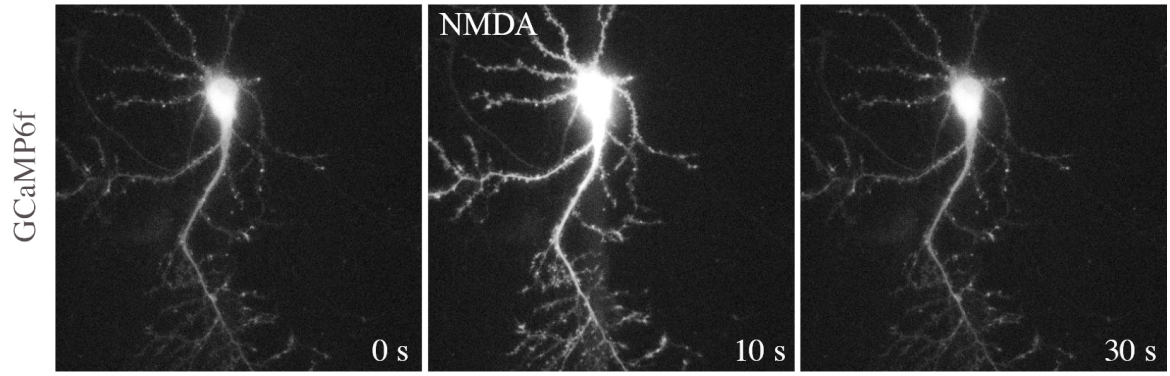

Figure 3.2 A GCaMP6f transfected cultured hippocampal neuron transiently and locally treated with 10 μM NMDA using a microfluidic pipette positioned adjacent to the soma

3.4 LOCAL AGONIST APPLICATION

In study IV we utilized a recirculation microfluidic based approach to apply ligands to single cells (Ainla et al., 2012). This was particularly beneficial when studying sparsely distributed GCaMP6f transfected neurons for several reasons: endogenous factors such as glycine were not rinsed out as when using perfusion, temperature control of bath solution was made simple, the treatment was confined and could be rapidly regulated in time, therefore unspecific Ca^{2+} effects of clamping the network function could be avoided and multiple cells on the coverslip could be treated in sequence. Moreover, the multi-well design allowed us to treat the same cell with different solutions and to do a paired comparison of the Ca^{2+} responses. To correct for possible variations in agonist delivery between and within pipettes we usually performed each experiment with a minimum of four individual pipettes, during at least four different days and performed a number of control experiments. Based on the specific experiment at hand we either used an equal amount of pipettes loaded with the same solutions and compared the cellular Ca^{2+} response to the treatment applied from different wells, or loaded the solutions into different pipettes and did an un-paired comparison of the responses. For our main findings we used both these strategies as controls.

3.5 SUPER-RESOLUTION IMAGING

Fluorescence microscopy techniques have traditionally been inherently limited in their resolution to approximately 200-250 nm in the lateral directions and 500-700 nm in the axial direction. This is due to the diffraction limit of light which infers that any imaging of a fluorescent molecule, no matter how small, will be blurred and enlarged according to the point spread function of the microscope. The diffraction limit given by Abbe's law states that for two objects to be clearly resolved in an optical system, the distance between them need to be at least half the wavelength of light i.e. ~200 nm for visible light. Cellular structures that are on the scale of micrometers, such as cell bodies or even the gross morphology of neuronal branches, can be studied at length with conventional microscopy techniques. The diffraction limit, however, renders it very hard or even impossible to image some of the most dynamic and complex neuronal structures such as dendritic spines, axons or presynaptic terminals, due to the lack of a higher spatial resolution. Simply put, everything at the scale near or below the diffraction limit will appear blurry. In recent years novel ideas on how to circumvent the diffraction limit have been exploited yielding a fast growing number of techniques that have vastly improved the spatial resolution in fluorescence microscopy. Three pioneers in the development of this field termed super-resolution microscopy, Stefan Hell, Eric Betzig and Willam E. Moerner, were for their achievements awarded with the Nobel Prize in Chemistry 2014 (For review see Hell Nobel lecture: 2015).

In study II we wanted to investigate the synaptic localization of Norbin in relation to key elements in dendritic spines. In study IV we set out to study the relationship between the NMDA receptor and the Na^+,K^+-ATPase in dendrites and dendritic spines at a single molecule level. Dendritic spines are around 0.5-2 μm long, have a spine head which is ~500 nm wide and a spine neck which is 50-150 nm wide. We therefore employed recently developed techniques that substantially improve the spatial resolution in fluorescence microscopy up to approximately one order of magnitude.

Though these novel techniques are highly varying in practice the ones employed in this thesis essentially share the same basic principles to improve resolution. They all rely on toggling fluorescent molecules between non-fluorescent dark OFF states and fluorescent ON states. This can be done in various ways which either rely on specific properties of the fluorescent molecules themselves or novel instrumentation and mathematical post-acquisition analysis. The techniques that were used here will be briefly described below.

3.5.0 Stimulated emission depletion microscopy (STED)

The idea that the principle of stimulated emission depletion could be used to circumvent the diffraction limit of light was first proposed in a theoretical paper and later on demonstrated in 2000 (Hell, 2015). Since then STED has been implemented to answer a variety of questions in neuroscience related to synaptic protein distribution as well as the structure and dynamics of dendritic spines (Blom et al., 2011; Blom et al., 2013; Willig et al., 2014; Nägerl et al., 2008; Nair et al., 2013). In STED, a second often donut shaped laser beam is superimposed on the excitation laser beam (Fig. 3.3). If the intensity of the donut shaped depletion laser is sufficiently high it will suppress the fluorescence caused by the excitation laser by forcing excited molecules to their ground state before emission occurs, a phenomena named stimulated emission depletion. Due to the shape of the superimposed laser the depletion will occur everywhere except in a small spot at the focal point when driven to saturation. This residual "sharpened" emission spot can in theory be made arbitrarily small meaning that STED can improve resolution down to the molecular scale. In practice however, the resolution is commonly around 40-50 nm in a fluorescently labeled biological sample. By scanning the excitation and superimposed depletion laser across the sample, high resolution images of labeled proteins in cellular structures can be detected without any need for post-acquisition analysis.

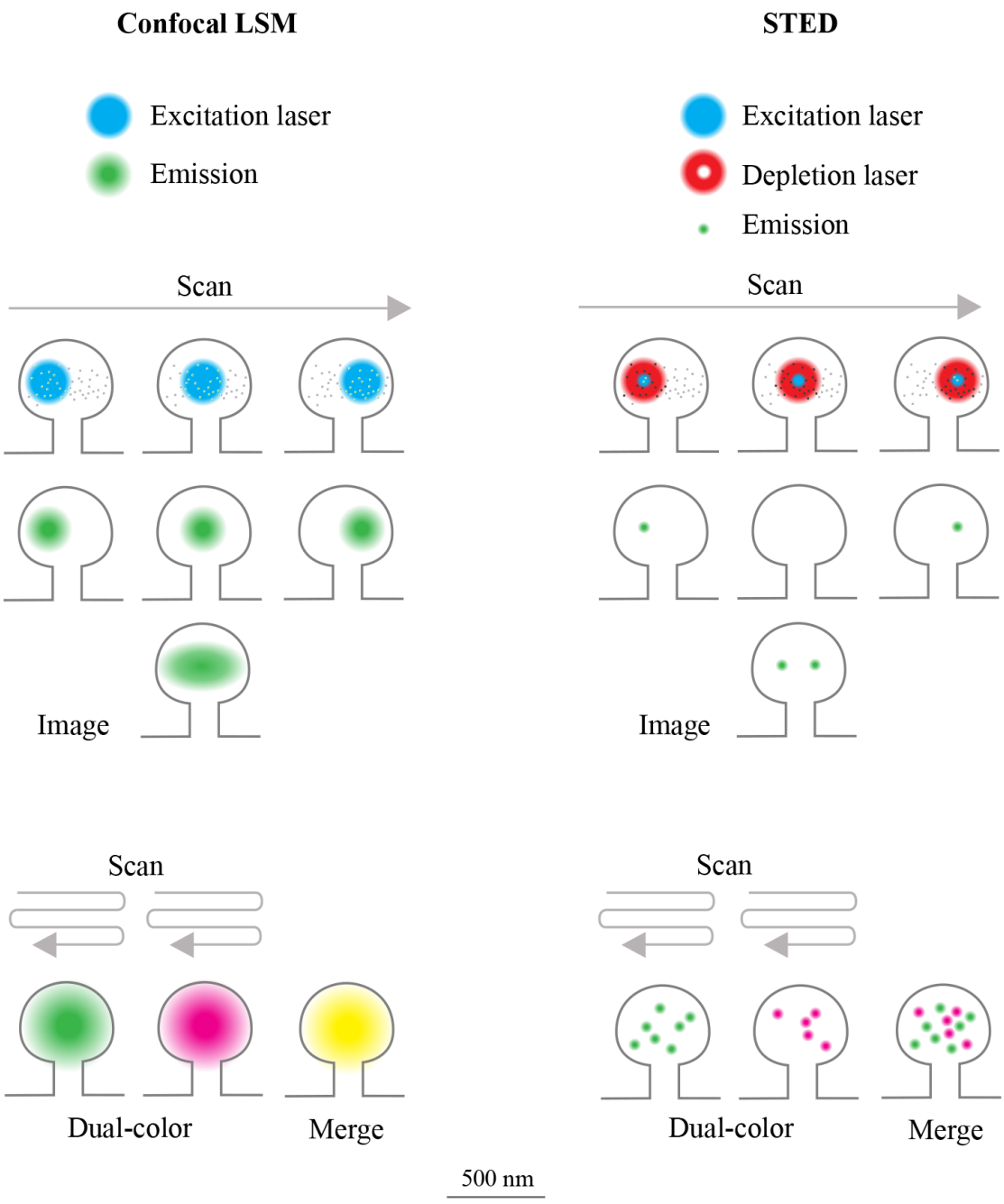

Figure 3.3 An illustration of conventional confocal laser scanning microscopy (LSM) (left), and stimulated emission depletion (right) of a dendritic spine.

3.5.1 Structured illumination microscopy (SIM)

Super-resolution structured illumination microscopy (SIM) is a widefield technique that has gained broad interest in part due to its ease of use. SIM does not depend on any specific photophysical features of the fluorescent molecules (unlike STED, PALM and STORM) and is compatible with conventional fluorescent proteins and dyes. Meaning that any fluorescently labeled sample can be imaged with SIM. This in combination with the possibility of performing multicolor and live 3D super-resolution imaging have made SIM an attractive candidate. SIM, in its basic implementation, increases the lateral resolution by approximately a factor of two, to 100 nm in the lateral direction and 300 nm in the axial direction (Gustafsson, 2000). The increase in resolution is achieved by illuminating the

sample with a well-defined high frequency grating (structured) pattern. The super-imposed grating pattern will generate an interference pattern with fine biological structures in the sample, so called moiré fringes. This allows high frequency information in the sample to be shifted into low-frequency information which can be detected by the camera. In practice these interference images can be generated by introducing a rotating periodic grid into the excitation field thus creating multiple stripy images. Based on the known parameters of the illumination pattern and the detected moiré fringes in the images, high frequency information in the sample can be decoded through mathematical post-acquisition analysis.

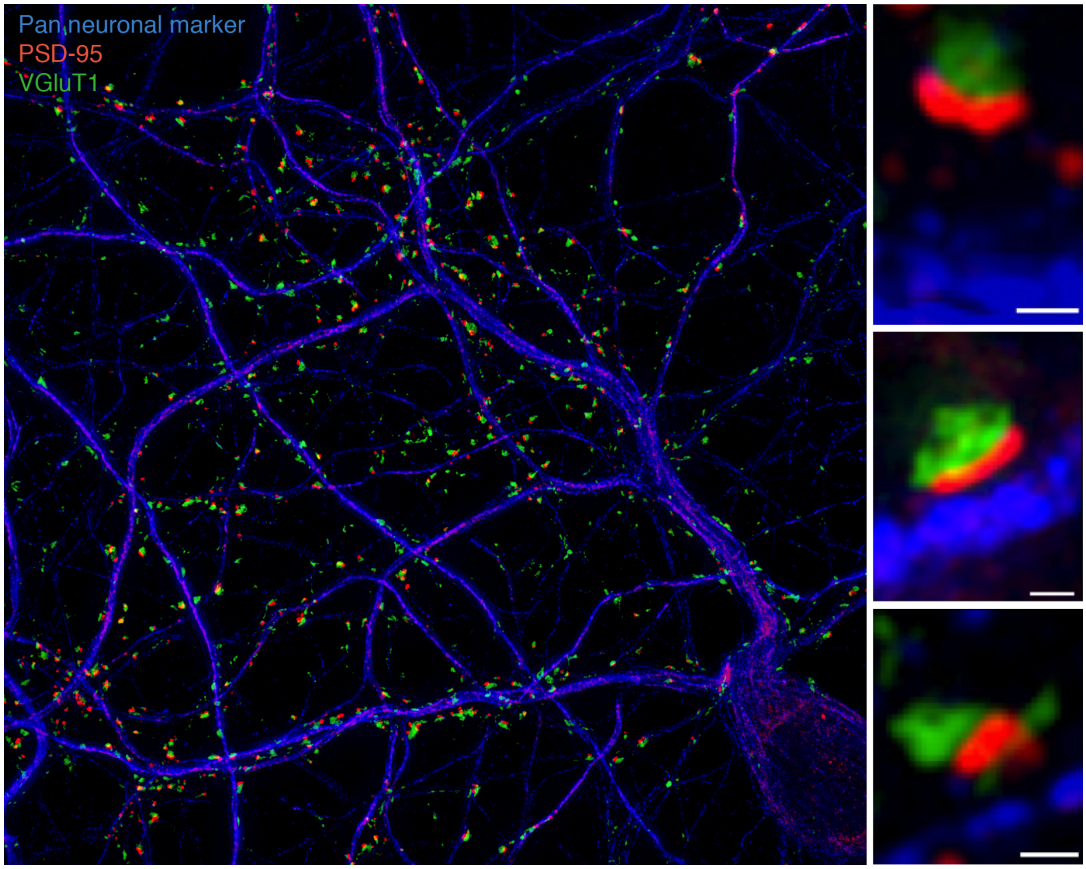

Figure 3.4 A structured illumination microscopy (SIM) image of a hippocampal neuron labeled for Pan neuronal marker, Postsynaptic density protein 95 (PSD-95) and VGluT1. Presynaptic termini and postsynaptic densities are clearly resolved and the synaptic cleft between them can be visualized. Scale bar = 400 nm.

3.5.2 Single molecule localization microscopy (PALM & STORM)

Photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) are two different acronyms essentially based on the same principle, yet they were developed in parallel utilizing different types of fluorescent molecules (Betzig, 2006; Rust et al., 2006). PALM was first implemented using photoactivatable fluorescent proteins while STORM was first demonstrated in a study using fluorescent synthetic dyes. PALM & STORM, also referred to as single molecule localization microscopy (SMLM), takes advantage of the time domain to separate the detected emission from fluorescent molecules from each other, more distance than the diffraction limit.

Although inherently limited by the diffraction limit, SMLM exploits the fact that single emitting molecules can be localized with high precision simply by finding the center coordinate

in the image of the fluorescent molecule. The obstacle in precisely determining the position of fluorescent molecules in labeled biological samples is that thousands of proximal molecules are simultaneously fluorescing, rendering them indistinguishable from one another at the detector. SMLM is achieved by separating the fluorescence emission events in the time domain by only allowing a random sparse subset of molecules in the sample to simultaneously emit. This can be achieved by using photoswitchable fluorescent molecules which can be randomly toggled between short-lived fluorescent ON and long-lived non-fluorescent OFF states by using widefield illumination. If only a sparse subset of the fluorescent molecules are simultaneously in an ON state and detected, they can be localized with high precision (Fig. 3.5). The precision is inversely proportional to the square root of the number of detected photons and is commonly around 10-20 nm in the lateral direction in a biological sample. By repeating the activation-detection-localization sequence of randomly activated molecules in the sample thousands of times, a high resolution image of the full ensemble of molecules can be reconstructed (Huang et al., 2009).

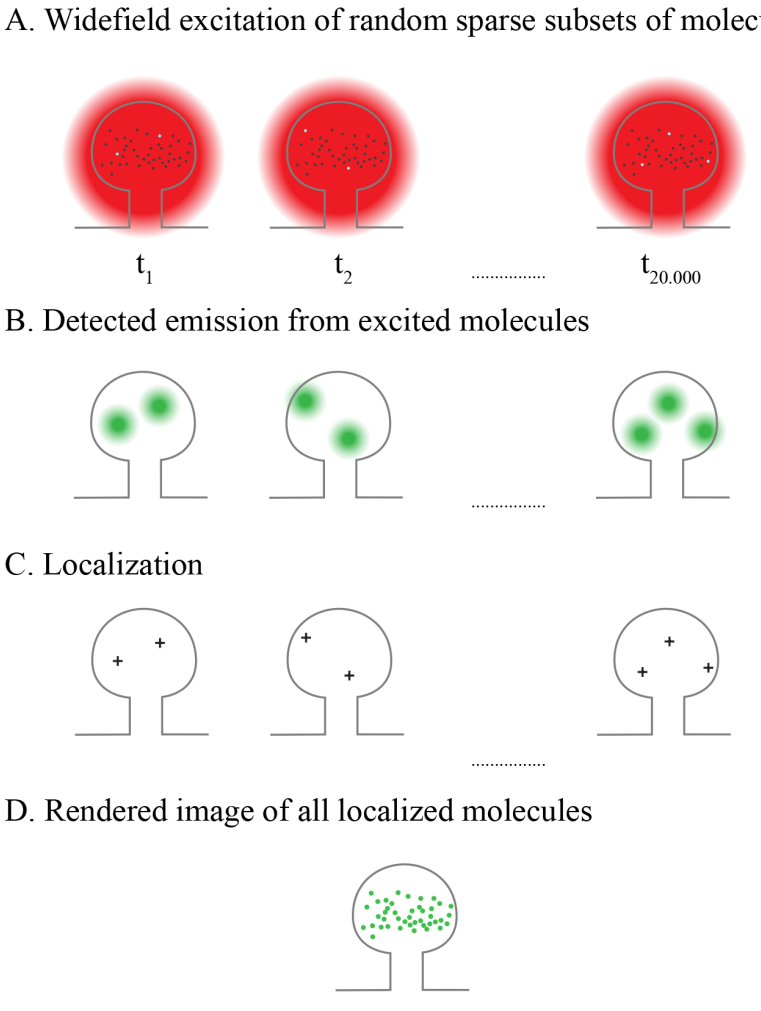

Figure 3.5 An illustration of single molecule localization microscopy (PALM and STORM).

Fluorescent photoswitchable proteins that are compatible with SMLM are e.g. photoactivatable GFP (PA-GFP) and mEOS (Patterson & Lippincott-Schwartz, 2004; McKinney et al., 2009). In study IV we employed direct stochastic optical reconstruction microscopy (dSTORM) (Heilemann, 2008). This version of SMLM uses a simple approach for the antibody labeling procedure with a single photoswitchable fluorescent antibody tagged

to the protein of interest. To facilitate effective photoswitching and dye stability the samples were embedded in an oxygen-scavenging system during image acquisition. We used a buffer consisting of catalase, glucose oxidase from aspergillus niger and cysteamine, which was reported to optimize the photoswitching of Alexa-647 and Atto-488.

3.5.3 Quantifying and interpreting super-resolution data

In the two studies using super-resolution imaging we employed two different strategies to quantify our data. In study II where we studied the synaptic localization of Norbin in relation to key elements and interacting proteins in dendritic spines we used Pearson's correlation coefficient as a measure of similarity in protein distributions (Dunn et al., 2011). Pearson's correlation coefficient is broadly implemented to quantify the degree of colocalization in biological studies not only in super-resolution based imaging. We used this approach first and foremost based on the resolving capability of SIM. Though the improvement in resolution gave significantly more spatial information than the confocal laser scanning microscope, still the appearance in particular with expressed proteins were rather continuous, as opposed to discrete. We therefore decided to use correlation analysis rather than a distance measuring based approach. Pearson's correlation coefficient quantifies the linear correlation between two image channels and gives values ranging from +1 to -1 where +1 means a high spatial correlation, 0 means that they are uncorrelated and -1 means that the distributions are anti-correlated

In study IV we used a nearest neighbor approach (Blom et al., 2012) to quantify the physical distances between single molecules as the coordinate for each molecule is given by the method. We determined the distance of each fluorescent molecule in one channel to the nearest fluorescent molecule in the other channel. Thereby ignoring all molecules located further away. Since the molecules are localized with a precision of 10-20 nm, this strategy is useful as an approach to investigate the premise of protein-protein interactions.

3.6 PROTEIN TRAFFICKING

3.6.0 Fluorescence recovery after photobleaching (FRAP)

Despite the highly organized distribution of proteins in cells, at the single molecule level few membrane and cytosolic proteins are firmly anchored for extended durations. Rather they move passively by e.g. diffusion or by active transport. FRAP has been extensively used to characterize ensemble diffusion of both membrane-bound receptors and cytosolic proteins and was initially demonstrated as a tool in the study of receptor mobility by Axelrod (Axelrod et al., 1976). The typical experiment is performed on fluorescently tagged receptors.

FRAP is achieved by photobleaching fluorescent molecules in a defined region using a high intensity laser and monitoring the fluorescence recovery over time. The fluorescent molecules in the bleached region will be irreversibly quenched, meaning that any recovery in fluorescence is due to an exchange with fluorescent molecules from outside of the bleached region. By analyzing the recovery profile, the fraction of immobile and mobile proteins can be quantified (Fig. 3.6). If a protein is completely free to diffuse, e.g. a small non-interacting inert protein like GFP itself, the recovery in fluorescence will be both fast and complete. In addition, by extracting the half-time of recovery ($t_{1/2}$), i.e. the time point at which half of the

final recovered fluorescence ($I_\infty/2$) occur, the diffusion constant can be approximated assuming a well-defined geometry of the bleached region.

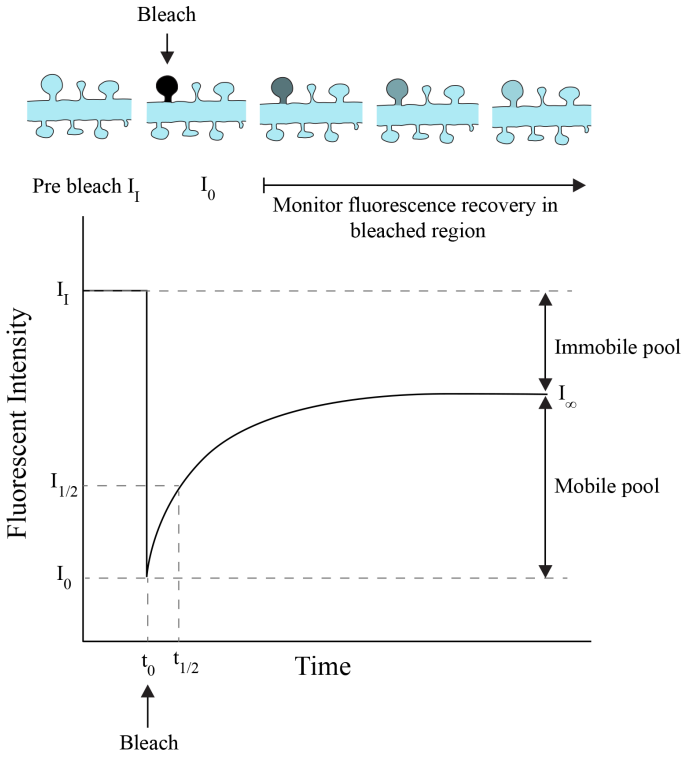

Figure 3.6 An illustration of a FRAP experiment in a spine. The fluorescent molecule is bleached and the recovery in fluorescent intensity is monitored over time.

3.6.1 Internalization - antibody feeding

In study IV we employed an alternative method to biotinylation to study NMDA receptor endocytosis using antibody live-labeling of surface receptors, sometimes to referred to as antibody feeding (Arancibia-Cárcamo et al., 2006). NMDA receptors are expressed in neurons as well as astrocytes and glia cells and we found them to be widely distributed across astrocytic processes in our primary neuronal cultures. Therefore we used an imaging based approach to potentially be able to observe small endocytic effects against a high background level of cell surface receptors in the culture and limit the observations to neurons specifically.

The basic idea is to, under live conditions, label the cell surface pool of the receptor of interest with an antibody which recognizes an extracellular epitope or tag of the receptor. After a labeling step, cells can be exposed to various experimental conditions and subsequently fixated. The assumption is that the receptor will be internalized in complex with the antibody similar as to if the receptor would internalize under physiological conditions. By incubating cells with one secondary antibody for the pool of receptors still on the cell surface prior to permeablization and another secondary antibody for the internalized pool of receptors post permeabilization, with a blocking step in between to avoid binding of the second secondary antibody to the pool of receptors on the surface, a ratio of internalized vs non-internalized receptors can be evaluated (Fig. 3.7). The critical step is to verify that the second secondary antibody will not bind to the antibody epitopes still available on the cell surface. We used $F(ab')_2$ fragments to achieve this blocking and titrated the amount and time needed

to under non-permeabilized conditions minimize the signal from the second secondary antibody.

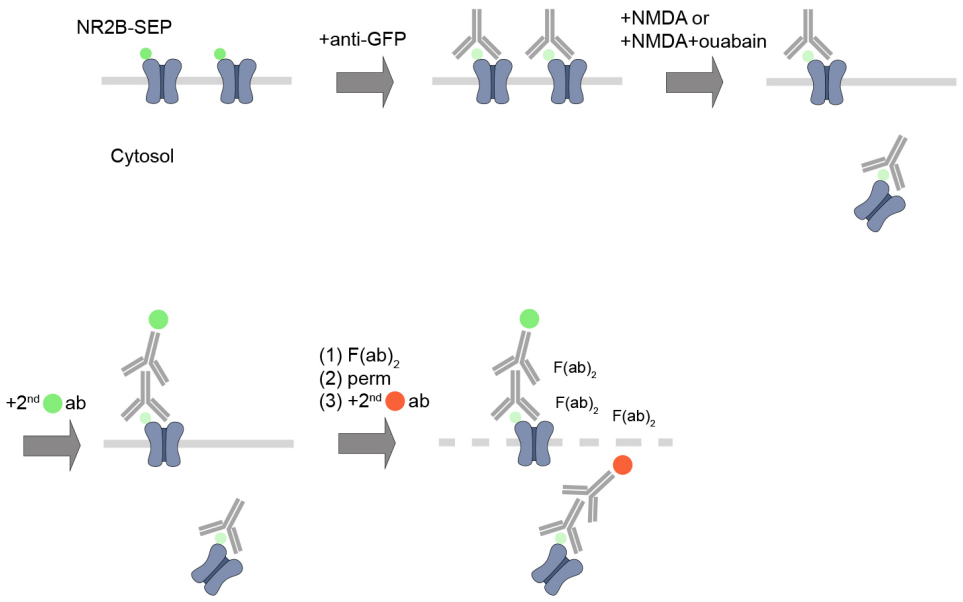

Figure 3.7. An illustration of an antibody feeding experiment to study receptor internalization.

4 SUMMARY

4.1 STUDY I - NORBIN AS A POSITIVE MODULATOR OF MGLUR5 ACTIVITY

The large group of mGluRs are widely distributed throughout the brain. Due to their diverse distributions and their modulatory role in neuronal function they are becoming increasingly interesting as pharmacological targets (Niswender & Conn, 2006). Balanced mGluR5 activity is key to normal brain function. Both hypo- and hyperactivity of the receptor have been implicated in multiple pathological states of the CNS. mGluR5 plays an important role in synaptic plasticity and we therefore screened for endogenous regulators.

We identified Norbin as an interacting molecule to mGluR5 intracellular C-terminus in a yeast two-hybrid screen. GST pull-down and co-immunoprecipitation studies confirmed an interaction between mGluR5 and Norbin. Two regions of the membrane proximal region of the C-terminus of mGluR5 were found to interact with the C-terminus of Norbin. The distribution of Norbin was found to largely overlap with previous reports on the overall distribution of mGluR5 in mouse brain, with abundant expression in e.g. hippocampus. Further, the expression of Norbin was located to dendritic and postsynaptic regions.

Activation of mGluR5 triggers the generation of IP_3 which activates IP_3Rs leading to the release of Ca^{2+} from the endoplasmic reticulum. To study whether the interaction between Norbin and mGluR5 could affect cell signaling, we co-transfected cells with mGluR5 and Norbin and studied the inositol phosphate levels. Compared to cells transfected with mGluR5 alone, the inositol phosphate hydrolysis levels increased, indicating an upregulation of mGluR5 activity. To test whether Norbin had an impact on mGluR5 mediated Ca^{2+} signaling, we performed live cell Ca^{2+} imaging experiments in HEK293 cells stably transfected with mGluR5 and transiently co-transfected with Norbin-GFP. Application of the group 1 mGluR specific agonist DHPG (10 μM) immediately triggered Ca^{2+} oscillations both in cells only expressing mGluR5 and in cells co-expressing Norbin. These oscillations decreased in amplitude and frequency over time, suggesting a desensitization of the receptor, possibly as an effect of internalization. However, several differences were identified between the cells expressing both mGluR5 and Norbin compared to cells expressing mGluR5 alone. Both the number of peaks, oscillatory duration and the number of cells that responded to DHPG was increased in cells co-expressing Norbin, indicating that Norbin plays a role in mGluR5 receptor activity and consistent with the observation of the increase in hydrolysis of inositol phosphate. The Ca^{2+} oscillation properties generated by mGluR5 activation are at least in part determined by the number of receptors expressed at the cell surface (Nash et al. 2002). We studied whether Norbin increased the plasma membrane expression of mGluR5 using biotinylation. mGluR5 cell surface expression was increased in Neuroblastoma N2A cells co-transfected with mGluR5 and Norbin whereas it was decreased in primary cultured cortical neurons five days after transfection with interfering RNA (ShRNA) targeting Norbin. Norbin overexpression did not increase the cell surface abundance in N2A cells expressing either of two different Norbin-binding deficient mutants of mGluR5 (mGluR5-mut1 and mGluR5-mut1/2). The positive regulation of mGluR5 mediated Ca^{2+} oscillations were further abolished in HEK293 cells expressing these mutants.

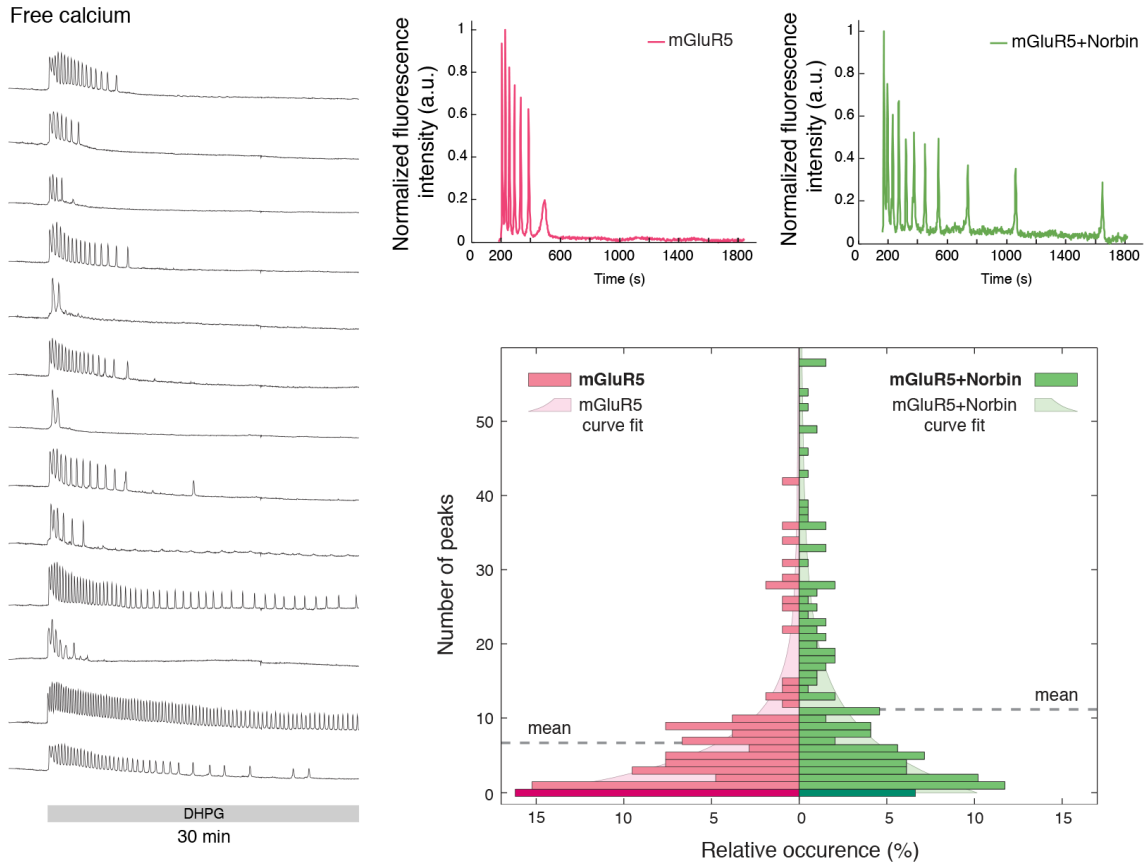

Figure 4.1 HEK293 expressing mGluR5 treated with DHPG display Ca^{2+} oscillations that decrease in frequency and amplitude over time to varying degrees (left). Cells overexpressing Norbin (green trace and bars) showed prolonged oscillations compared to control cells (pink trace and bars). The relative occurrence in the number of peaks displayed by mGluR5 or mGluR5+Norbin expressing cells are shown in the diagram.

It was previously shown that a prenatal unspecific knockdown of the Norbin gene resulted in early embryonic lethality, conceivably due to the knockdown of its expression in chondral or bone tissue (Moshizuki et al., 2003). On order to study the effect of Norbin on mGluR5 function in vivo, we generated conditional Norbin knockout mice with a postnatal forebrain specific deletion by crossing $Ncdn^{Flox/Flox}$ mice with CamKIIα-iCre transgenic mice. The cell surface level of mGluR5 was reduced in Norbin KO ($Ncdn^{Flox/Flox;iCre}$) compared to WT ($Ncdn^{Flox/Flox}$), while total mGluR5 levels where unaffected, suggesting a role for Norbin in the trafficking or steady state cell surface expression of the receptor.

mGluR5 activation can trigger both LTP and LTD (Huber et al., 2000, Lu et al, 1997; Jia et al., 1998; Bortolotto et al., 2005). We therefore studied field excitatory postsynaptic potentials (fEPSP) in hippocampal slices from Norbin KO and WT mice during chemical induction of LTD using the group 1 mGluR agonist DHPG. Application of 100 µM of DHPG for 5 minutes in slices from WT mice resulted in LTD as measured in a reduction of fEPSP slopes. The DHPG-induced LTD was impaired in slices from Norbin KO mice. mGluR5 stimulation can trigger activation of extracellular signal-regulated kinase (ERK), which is required for LTD induction (Volk et al., 2006). Norbin was found to increase the levels of phosphorylated ERK minutes following DHPG application. mGluR5 is also associated to LTP. Mice with a genetic deletion of mGluR5 display impaired CA1 LTP (Lu et al, 1997; Jia

et al., 1998; Bortolotto et al., 2005). Norbin KO mice showed a deficit in CA1 LTP, similar to the observations made in mGluR5 KO mice.

Pre-pulse inhibition (PPI) of a startle is the ability of a weaker non-startling prestimulus (pre-pulse) to suppress the response of a subsequent stronger startling stimulus so that e.g. a low noise can suppress the startle reflex to that of a higher subsequent noise. Deficits in this plastic behavioral PPI response is seen in patients with schizophrenia and is believed to be an important feature of the disease (Powell et al., 2009). Aberrant PPI is thought to reflect a deficiency in sensorimotor gating and expose an impairment in filtering relevant information from sensory input. Both NMDA NR1 KO and mGluR5 KO mice display substantial disruption of PPI (Powell et al., 2009). In view of this robust feature of mGluR5 hypofunction, we compared the PPI in response to acoustic stimuli between Norbin KO mice and WT mice. In agreement with mentioned studies linking PPI to mGluR5 hypofunction, the PPI response was found to be reduced in Norbin KO mice compared to WT controls.

The NMDA receptor antagonist MK-801, which blocks the NMDA pore, can increase locomotor activity in mice. Pretreatment with the mGluR5 specific antagonist MPEP can augment the MK-801 triggered increase in locomotor activity (Pietraszek et al., 2006). Treatment with MPEP alone however does not induce hyper locomotor activity suggesting that mGluR5 plays an important role in enhancing NMDAR activity in vivo. Norbin KO mice, similar to mice pre-treated with MPEP, showed a substantial increase in locomotor activity after treatment with MK-801. And importantly, abolished the additive effect of MPEP seen in WT mice treated with MK-801.

Several mGluR5 interacting proteins have been identified such as Homer, Shank and the Ca^{2+} binding protein calmodulin. Of these the Homer family has been extensively studied and shown to play an important regulatory role in mGluR5 function. While Homer binds to the membrane distal part of the receptor, Norbin was found to interact with the membrane proximal region of mGluR5. Two regions of mGluR5 were found to be critical for the interaction with Norbin. These regions were found to also partly overlap with the binding of calmodulin.

In summary, Norbin binds to the C-terminus of mGluR5, facilitate cell surface expression and positively modulate mGluR5-mediated signaling. Norbin KO mice show impairments in mGluR5 mediated LTP and LTD and a behavioral phenotype corresponding to hypofunctional mGluR5 activity.

4.2 STUDY II - SUBCELLULAR EXPRESSION OF NORBIN ON THE NANOSCALE

In study I, we identified Norbin as a novel regulator of mGluR5 activity. However, the mechanism on how Norbin increases the cell surface expression of mGluR5 was not determined. We therefore investigated the subcellular localization of Norbin to elucidate where the regulation of mGluR5 might occur.

mGluR5 is abundantly expressed in hippocampus and is found mainly in dendrites and spines with an accumulation in the perisynaptic annulus surrounding the postsynaptic density (Lujan et al., 1996). We therefore studied the subcellular expression of Norbin in primary

hippocampal neurons. In agreement with previous reports we found Norbin to be widely expressed across the soma, dendrites and dendritic spines with a cytosolic appearance. To determine if Norbin, despite appearing highly homogenous, was enriched in subcellular locations we co-expressed Norbin fused to GFP with an inert empty vector fluorescent protein. Images comparing the expression patterns highlighted an enrichment of Norbin in dendritic spines and a lower abundance in axons compared to the empty vector expression level.

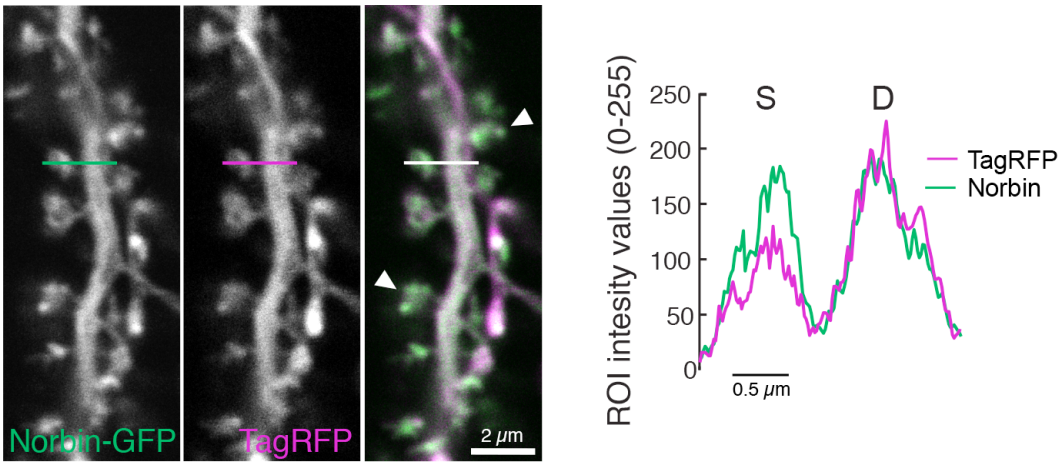

Figure 4.2 Norbin is enriched in hippocampal dendritic spines (S), compared to the dendrite (D) and compared to the inert fluorescent protein TagRFP.

FRAP experiments in dendrites and spines comparing the half-time of recovery and the mobile pool of Norbin with the empty vector, further suggested that Norbin was stabilized in dendritic spines, yet highly mobile compared to other proteins more firmly anchored in the postsynaptic density such as PSD-95 (Kuriu et al., 2006).

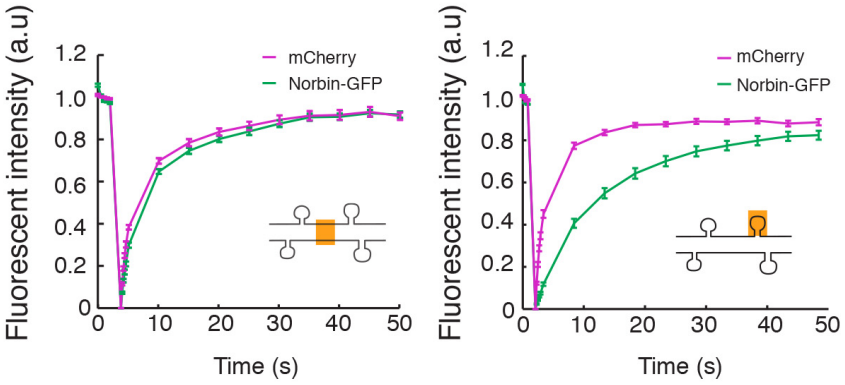

Figure 4.3 FRAP experiments in dendrites and spines show that Norbin moves similar to that of an inert fluorescent protein (mCherry) in dendrites (left) but has a reduced mobility in dendritic spines (right).

Dendritic spines are small actin-rich protrusions with diverse morphology that receive and partly compartmentalize synaptic input. Their micrometer size makes them unavailable to spatial investigations using conventional fluorescence based methods. Super-resolution microscopy techniques enhance the resolution of fluorescence microscopy up to an order of

magnitude, in some applications to the molecular scale. We were mainly interested in studying the expression of Norbin in relation to the larger elements in the dendritic spine. We therefore used SIM, which gives an intermediate improvement in resolution of ~100 nm, and STED which can give ~40 nm resolution in the lateral directions, to study the localization of Norbin in relation to postsynaptic elements, while also comparing the two techniques. Considering that both antibodylabeling and overexpression of proteins have distinct advantages and setbacks we used both labeling techniques and compared the results.

STED works well with numerous synthetic fluorescent dyes but is limited in its performance on fluorescent proteins. We therefore used STED to study the endogenous expression of antibody-labeled Norbin and PSD-95. PSD-95 is located immediately beneath the active site in the spine head and interacts directly with the ionotropic AMPA and NMDA receptors, but only indirectly with mGluR5 which is perisynaptically accumulated. Chains of PSD multimers such as Homer, Shank and PSD-95 links mGluR5 to the NMDA receptor.

We found that Norbin mainly surrounded PSD-95, and they showed little spatial overlap. Experiments using SIM gave similar results with overexpressed Norbin and PSD-95. There were clear differences in the observed distributions of endogenous and overexpressed Norbin. Antibody-labeled Norbin appeared as small clusters seen throughout the dendrites and spines. Overexpressed Norbin had a more continuous appearance. These differences were seen also using conventional microscopy methods and are most likely a result of the fundamental differences in labeling techniques. The antibodylabeling procedure can result in insufficient crosslinking and subsequent unwanted extraction of proteins, paraformaldehyde crosslinking induced masking of antibody epitopes and possibly steric hindrance of antibody diffusion. Overexpression can naturally result in uncontrolled levels of expression, leading to a dysregulated localization e.g. due to a saturation of interactions with endogenous partners. In spite of these differences the general relation of Norbin to PSD-95 were consistent between STED and SIM of endogenous and overexpressed Norbin respectively. The limited spatial correlation was not observed using the diffraction limited confocal microscope in which Norbin and PSD-95 displayed a complete spatial overlap.

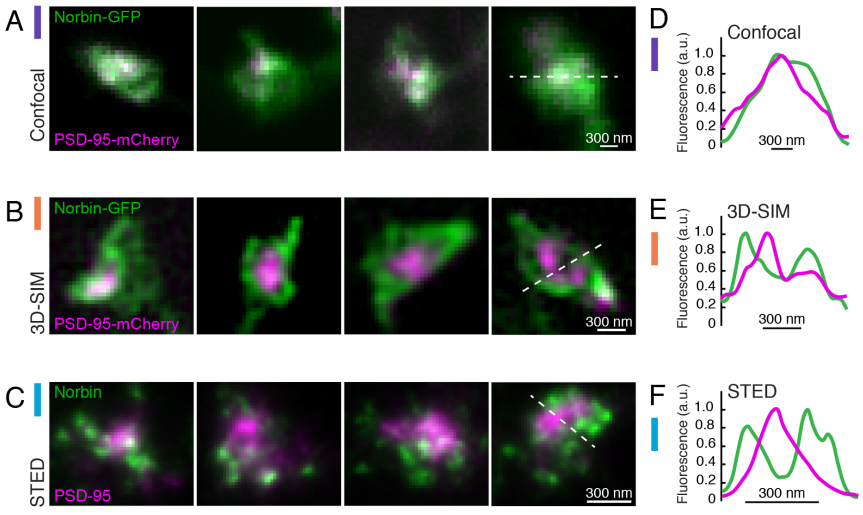

Figure 4.4 A comparison of labeled Norbin and PSD-95 in dendritic spines and imaged with confocal LSM (top), structured illumination microscopy (SIM) and stimulated emission depletion (STED) (bottom).

The overall similarity between the results from the STED and the SIM experiments, which demonstrated that SIM gave a sufficient improvement in resolution to resolve structural elements in the spine, suggested that we could use SIM to study the relation to other components in the spine. Actin is the predominant cytoskeletal component in the spine. It extends from the dendrite into the spine head and provides a structural support for the PSD (Hotulainen & Hoogenraad, 2010). Considering that the spatial correlation between Norbin and PDS-95 was limited, we studied the correlation with actin and found them, with the 100 nm lateral resolution, to overlap to a high degree. Although not quantified, Norbin and actin had similar distributions in dendrites. Co-immunoprecipitation experiments confirmed the results from the imaging experiments. Norbin was found to co-immunoprecipitate with actin but not with PSD-95. Western blot experiments in PSD fractions also showed that Norbin was not part of the PSD. Norbin did however show a high correlation with mGluR5 and the mGluR5 interacting partner Homer which is located in the deeper layers of the PSD (Dani et al., 2010).

These results indicate that Norbin can facilitate mGluR5 cell surface expression via a different mechanism than Homer, which acts as a PSD scaffolding molecule.

4.3 STUDY III – P11 AS POSITIVE MODULATOR OF MGLUR5 ACTIVITY

In this study, we show that p11 interacts directly with the C-terminus of mGluR5. Co-immunopreciptiation experiments in HEK293 cells overexpressing flag-tagged WT p11 vector and a mutated p11 unable to bind to AnxA2 indicated that AnxA2 is required for the interaction between p11 and mGluR5. GST-studies using a GST-p11-AnxA2 construct confirmed an interaction with mGluR5 in both cortical and hippocampal tissues. Mutating the mGluR5 C-terminal by replacing 5 residues (amino acids 836-840) with alanine abolished the binding of p11.

To study a potential effect of p11 on mGluR5 signaling we co-transfected cells with mGluR5 and p11 fused to GFP, or a control vector, and compared the response during application of the group 1 mGluR agonist DHPG in live cell Ca^{2+} experiments. As expected, cells responded with oscillating cytosolic Ca^{2+} levels that, as in study I, generally decreased in amplitude and frequency over time suggesting an activity-dependent desensitization. Cells overexpressing p11 had a slightly higher frequency in Ca^{2+} oscillations than control cells which could be suggestive of an increase in plasma membrane expression and/or altered phosphorylated state of mGluR5 (Nash et al., 2002; Lee et al., 2008). Furthermore, a mutated mGluR5, unable to bind p11 showed a reduction of the oscillatory frequency of mGluR5. These results suggest that p11 likely facilitate mGluR5 cellular function by positively regulating downstream signaling events. In agreement with the positive regulation of p11 on mGluR5 mediated Ca^{2+} signaling, biotinylation and immunocytochemical experiments showed that p11 and mGluR5 reciprocally facilitate one another's accumulation at the plasma membrane.

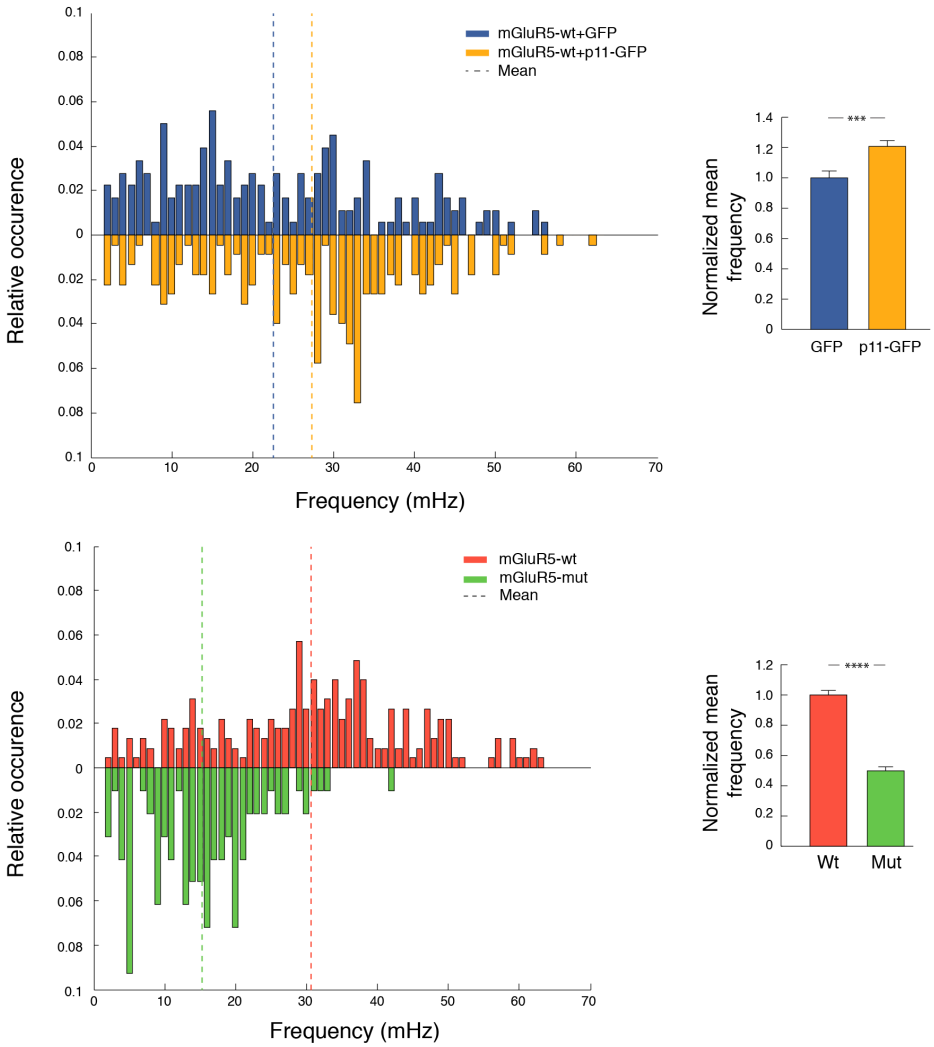

Figure 4.5 Ca^{2+} experiments in HEK293 cells expressing mGluR5 and treated with DHPG. P11 overexpression (yellow bars) increases the frequency of Ca^{2+} oscillations compared to control cells (blue bars). A mGluR5 mutant, deficient in binding p11 (green bars), show a reduction in the frequency of Ca^{2+} oscillations compared to wild-type mGluR5 (red). Cells were loaded with the Ca^{2+} sensitive indicators Fura-2 (top), or Oregon green 488 BAPTA-1 (bottom).

We next sought to investigate a potential role of p11-mGluR5 interaction in depression-like states. Imbalances in the activity of inhibitory and excitatory synaptic transmission have been implicated in the etiology of several CNS disorders. Both mGluR5 and p11 have been found to localize to glutamatergic and GABAergic cells. We therefore generated cell-type specific knockout mice with a deletion of mGluR5 or p11 in glutamatergic or GABAergic neurons by breeding floxed p11 and floxed mGluR5 mice with EMX-Cre (for genetic deletion in glutamatergic neurons) or GAD-Cre mice (for genetic deletion in GABAergic neurons).

A set of behavioral assays implemented to transiently infer depression-like behavior or despair such as immobility in response to tail suspension test (TST) and forced swim test (FST) were conducted. Both EMX-mGluR5 KO and EMX-p11 KO mice showed depression-like behavior triggered by FST and TST measured as degree of immobility, and displayed anhedonia as measured in the reduced sucrose consumption test (SPT). Inversely both GAD-mGluR5 KO and GAD-p11 KO mice showed antidepressive-like behavior in the FST, TST and SPT. These experiments demonstrate that both p11 and mGluR5 play a role in animal

models of depression and that genetic deletion of p11 and mGluR5 share overlapping phenotypes.

To test whether the previously reported anti-depressant effect of the mGluR5 specific antagonist MPEP could depend on an interaction between p11 and mGluR5, we used the novelty suppressed feeding test to explore depression-like behavior. The anti-depressant like effect of MPEP was observed in WT mice ($p11^{+/+}$) but was abolished in constitutive p11 KO ($p11^{-/-}$) mice. Both animals where p11 or mGluR5 had been genetically deleted in glutamatergic neurons (EMX-mGluR5 KO and EMX-p11 KO) responded with anti-depressive behavior to MPEP, similar to WT control animals.

In contrast, animals where p11 or mGluR5 had been genetically deleted in GABAergic interneurons (GAD-mGluR5 KO and GAD-p11 KO) showed baseline antidepressant-like behavior, with no further anti-depressant effect of MPEP. This blunted effect of MPEP persisted in mice where the mGluR5 or p11 expression was deleted in the parvalbumin (PV) positive subset of interneurons, suggesting that mGluR5-expressing PV-positive neurons play a dominant role in the anti-depressant effect exerted by MPEP. In vivo spike recordings in medial prefrontal cortex, a region implicated in depression, of WT mice showed that MPEP increased the firing rate of glutamatergic neurons, while it decreased the firing of GABAergic neurons. This suggests that the antidepressant effect of MPEP might correlate to a shift in the balance between excitatory and inhibitory activity.

In summary, p11 binds to the C-terminus of mGluR5 to increase receptor cell surface expression and increase the frequency of mGluR5 mediated Ca^{2+} oscillations. A genetic deletion of p11 in GABAergic, and more specifically PV-positive neurons, blocks the antidepressant effect of the mGluR5 antagonist MPEP.

4.4 STUDY IV - NMDA RECEPTOR REGULATION BY THE NA^+,K^+-ATPASE

There are three isoforms of the catalytic Na^+,K^+-ATPase α subunit expressed in the CNS: the α1 isoform which is commonly referred to as ubiquitous and expressed in diverse tissues, the α2 isoform predominantly expressed in astrocytes and the α3 isoform which is neuron specific. A genetic deletion of either of the α1, α2 and α3 subunits of the Na^+,K^+-ATPase leads to embryonic or early postnatal death (Moseley et al., 200). A study investigating the behavior in heterozygous mice where each of the Na^+,K^+-ATPase α alleles were genetically deleted, identified differences in behavioral deficits specific for each of the isoforms. Mice heterozygous in the Na^+,K^+-ATPase α3 and α2, but not α1 isoform show deficits in spatial learning and memory. Mice heterozygous in the Na^+,K^+-ATPase α3 display hyperactive locomotor activity while the α2 mice display decreased locomotor activity. Based on the Na^+,K^+-ATPase α3 specific phenotype, the NMDA receptor content was evaluated in α3 heterozygous and found to be reduced with 40% (Moseley et al., 2007).

The NMDA receptor and the catalytic Na^+,K^+-ATPase α subunit have previously been reported to co-immunoprecipitate in cerebellar tissue, suggesting they can form complexes in vivo (Akkuratov et al., 2015). The Na^+,K^+-ATPase specific ligand ouabain has been shown to protect from excitotoxicity in several studies, suggesting that the Na^+,K^+-ATPase might play a role in glutamate receptor overactivation (Wang et al., 2006; Golden & Martin, 2006;

Dvela-Lewitt et al., 2014). We therefore decided to investigate a potential functional relationship between these two membrane proteins.

We began by characterizing the distributions of NR2A and NR2B subunits in relation to the two Na^+,K^+-ATPase α isoforms expressed in neurons (α1 and α3) using super-resolution microscopy. We chose single molecule localization microscopy of endogenously antibody labeled protein as an approach to investigate the anatomical basis of a functional relationship in hippocampal neurons. As described in the methodology section, single molecule localization techniques can determine the position of single fluorescent molecules with a precision of 10-20 nm making it a feasible strategy to investigate the pre-requisite of protein-protein interactions in intact cells, i.e. to determine if the are close enough for a direct interaction to occur.

We pairwise immunolabeled cells with antibodies for either of the NR2 subunits and either of the Na^+,K^+-ATPase α isoforms. The NR2 subunits showed clustered distributions as expected from synaptic-like expression patterns. The Na^+,K^+-ATPase α isoforms in contrast displayed broader distributions along the postsynaptic membranes. Many molecules, often several thousands, were localized in a portion of a dendritic branch. To quantify the proximity at the single molecule level we chose to quantify the distance between the nearest neighbors. For every NR2 subunit we determined the distance to the nearest Na^+,K^+-ATPase α isoform. And for every Na^+,K^+-ATPase α isoform we determined the distance to the nearest NR2 subunit. We found both NR2 subunits to be in close spatial proximity to the Na^+,K^+-ATPase. In particular Na^+,K^+-ATPase α3 was closely positioned to the NR2 subunits. Every other NR2A or NR2B subunit had a Na^+,K^+-ATPase α3 isoform closer than 50 nm.

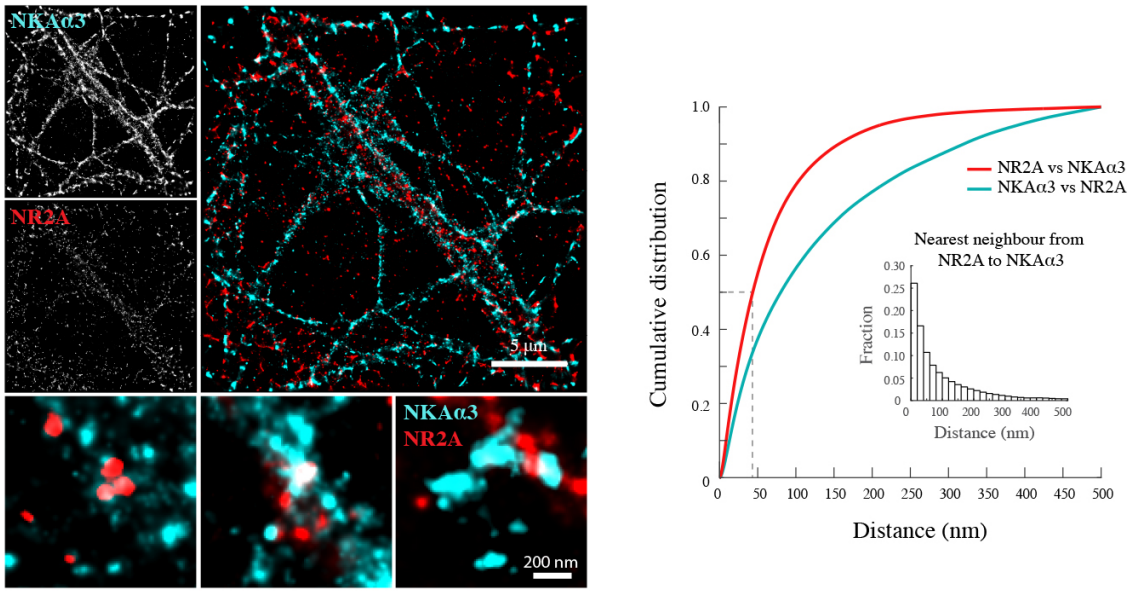

Figure 4.6. A reconstructed image from a direct stochastic optical reconstruction microscopy (dSTORM) experiment in a hippocampal neuron antibodylabeled for Na^+,K^+-ATPase α3 and NR2A. The cumulative distribution plot shows the distances from every NR2A to the nearest Na^+,K^+-ATPase α3 (red) and the distances from every Na^+,K^+-ATPase α3 to the nearest NR2A (blue) within a 500 nm radius.

The close spatial proximity of the NMDA receptor and the Na^+,K^+-ATPase, in combination with reports that ouabain can protect against excitotoxicity prompted us to test whether

Na^+,K^+-ATPase can influence NMDA receptor dependent Ca^{2+} influx which has been proposed to be a main contributor to Ca^{2+} mediated neuronal cell death after ischemic injury (Lipton & Rosenberg, 1994). The Na^+,K^+-ATPase specific ligand ouabain (a cardiotonic steroid) locks the catalytic isoform in a conformational state where it is unable to transport Na^+ and K^+. High concentrations of ouabain will effectively inhibit the Na^+,K^+-ATPase α1 and α3 and cause membrane depolarization. Concentrations in the low nanomolar range are however not expected to cause a rapid membrane depolarization.

We transfected primary cultured neurons with the genetically encoded Ca^{2+} sensor GCaMP6f and exposed cells locally to NMDA using a microfluidic device. The experiments were performed in the absence of extracellular magnesium to minimize unspecific effect on the NMDA receptor open probability due to possible ouabain-induced changes in membrane potential. Cells locally and repeatedly exposed to NMDA alone did not show a significant desensitization during repeated short-term application of NMDA. When low doses of ouabain was co-applied with NMDA the Ca^{2+} response however was immediately reduced.

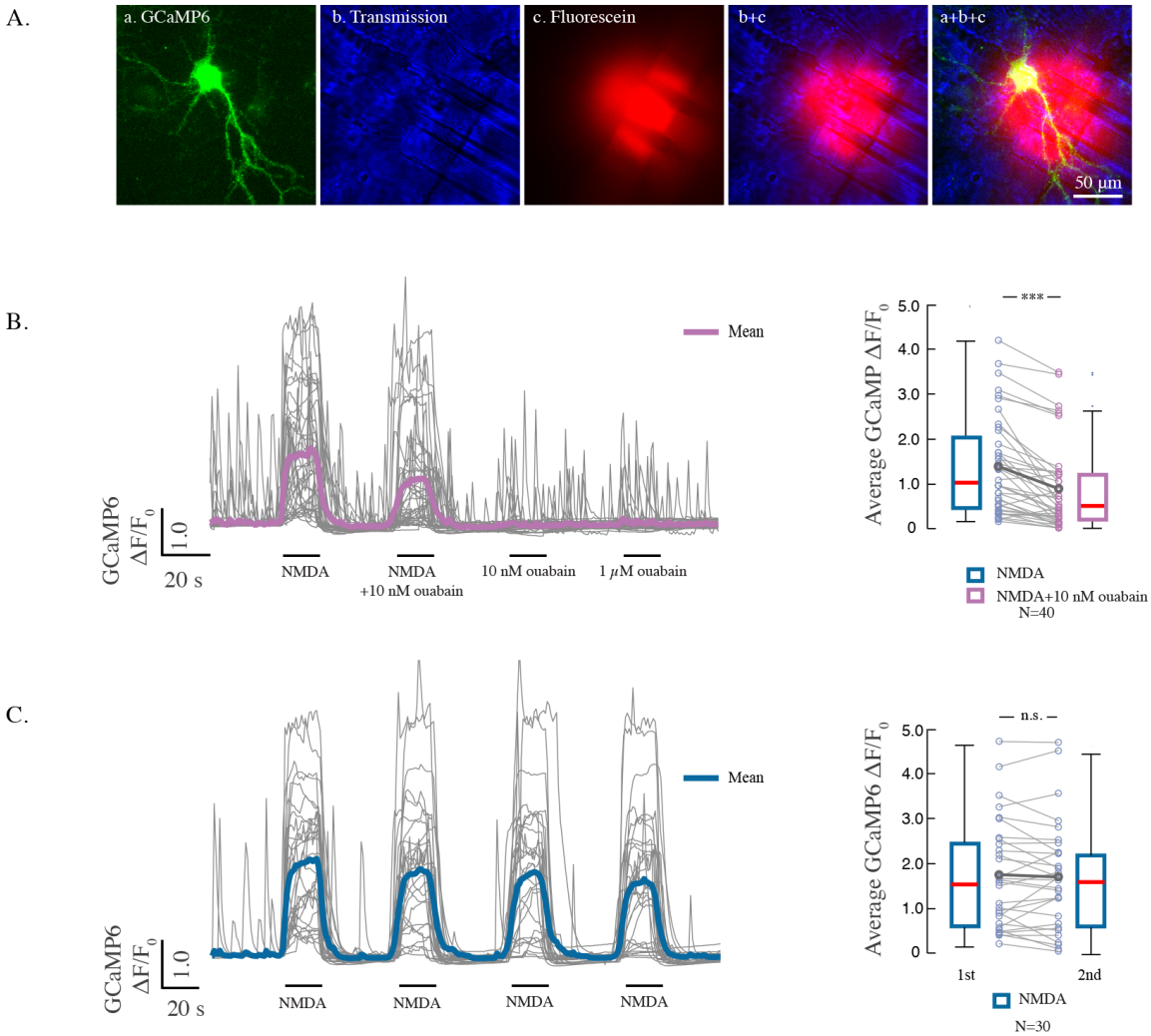

Figure 4.7 Ca^{2+} experiments performed in cultured hippocampal neurons during local stimulation A. An illustration with overlayed images showing a GCaMP6f transfected neuron (a) and the pipette tip (b) filled with fluorescein (c). B. 10 nM of ouabain attenuates the Ca^{2+} of the NMDA receptor, but has no effect on resting Ca^{2+} levels alone. C. Repeated application of NMDA alone does not desensitize the Ca^{2+} response, quantified between the 1st and 2nd NMDA pulse.

To test whether the effect was specific for NMDA receptor dependent Ca^{2+} influx, we studied the Ca^{2+} response in cells exposed to glutamate in the presence of the NMDA receptor competitive antagonist D-APV. Glutamate in the presence of D-APV would allow for a Ca^{2+} influx via ionotropic GluR2 subunit lacking AMPA receptors and kainate receptors as well as trigger a release of Ca^{2+} from the endoplasmic reticulum via metabotropic glutamate receptors. However, ouabain had no immediate bearing on the average Ca^{2+} influx during the short-term stimulation when the NMDA receptor was blocked. These results suggest that ouabain binding to the Na^+,K^+-ATPase regulate NMDA receptor mediated Ca^{2+} influx specifically. We did observe a gradual decrease in the Ca^{2+} response when 10 nM of ouabain was co-applied with glutamate and D-APV. The effect was however not as immediate as when NMDA receptors were activated. Interestingly, the Na^+,K^+-ATPase has been reported to interact with AMPA receptors (Zhang et al., 2009).

Results from equivalent Ca^{2+} studies in HEK293 cells, expressing only the Na^+,K^+-ATPase α1 isoform endogenously suggest that the effect of ouabain is limited to the neuron specific Na^+,K^+-ATPase α3 isoform. Cells expressing Na^+,K^+-ATPase α3 exogenously had a stronger Ca^{2+} response to NMDA compared to cells only expressing α1. In addition, ouabain had an effect only in cells co-transfected with Na^+,K^+-ATPase α3.

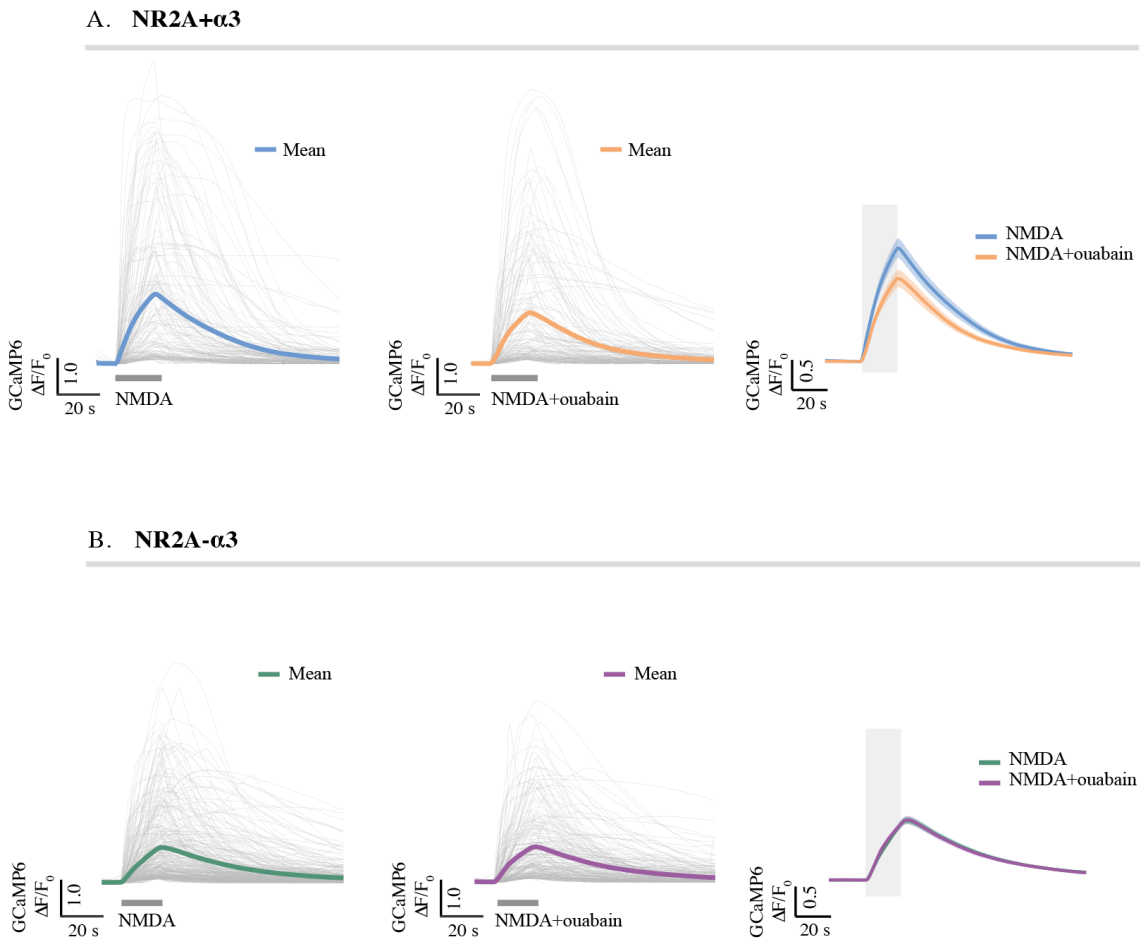

Figure 4.8 Ca^{2+} experiments performed in HEK293 cells expressing endogenous Na^+,K^+-ATPase α1 and exogenous NR1 and NR2A + α3 (top) and – α3 (bottom) and stimulated with 10 µM NMDA (left) or 10 µM NMDA + 10 nM ouabain (right). Cells with an exogenous expression of Na^+,K^+-ATPase α3 show a reduction in the NMDA triggered Ca^{2+} influx in the presence of ouabain. This effect is not observed in cells not expressing Na^+,K^+-ATPase α3.

Nanomolar concentrations of ouabain has been shown to trigger cell signaling pathways in a variety of organs and to serve cell protective effects (Aperia et al., 2016). In search for the mechanism underlying the effect of ouabain on NMDA receptor activity, we investigated whether the Src family of tyrosine kinases (SFK) could mediate the effect of ouabain. The SFK, in particular the SFK members src and fyn, has been shown to play a critical role in regulating NMDA receptor activity. Both NR2A and NR2B contain multiple tyrosine SFK regulated sites which control the open probability of the NMDA receptor (Salter & Kalia, 2004). Moreover, ouabain has been shown to regulate Src activity via the Na^+,K^+-ATPase (Tian et al., 2006). We therefore examined whether the SFK could play a role in the down-regulation of NMDA receptor activity induced by ouabain. However, we were unable to detect any differences in the effect of ouabain while inhibiting the SFK using the SFK specific inhibitor PP2.

NMDA receptors can undergo rapid endocytosis in primary culture, in particular NR2B (Lavezzari et al., 2004; Roche et al., 2001). We therefore also examined whether ouabain may induce rapid NR2B internalization. Short term treatment, 5 or 15 minutes, with ouabain did however not influence the endocytotic rate of the NR2B.

In summary, ouabain affects the Ca^{2+} influx through the NMDA receptor specifically via the Na^+,K^+-ATPase α3 isoform. We could not detect any role of SFK, any effect on the membrane potential or the internalization rate of the NR2B. Considering that the effect of ouabain was immediate and reversible, and that the NMDA receptor and the Na^+,K^+-ATPase can interact directly in combination with their close spatial relationship, indicate that the effect of ouabain might be via an allosteric effect caused by a ouabain dependent conformational change of the Na^+,K^+-ATPase α3.

4.5 DIFFERENTIAL DISTRIBUTIONS OF NA^+,K^+-ATPASE A1 AND A3 ISOFORMS – ONGOING PROJECT

In study IV we observed that not all neurons responded with an attenuated Ca^{2+} response during co-application with ouabain. Importantly, the rodent α3 isoform has a much higher affinity to ouabain than the rodent α1 isoform. We therefore hypothesized that the effect could be due to a cell type specific expression of the different Na^+,K^+-ATPase α isoforms. We made the assumption that there could be differences in the level of expression in excitatory glutamatergic neurons and in inhibitory γ-aminobutyric acid (GABA) expressing neurons. We immunolabeled primary cultured hippocampal cells with either of the α isoforms as well as VGluT1 (vesicular glutamate transporter 1) and GAD-65/67 (glutamate decarboxylase 65/67) as markers of excitatory and inhibitory cells respectively.

The Na^+,K^+-ATPase α3 isoform was predominantly expressed in cells that also stained positive for GAD-65/67. Conversely, the Na^+,K^+-ATPase α1 isoform showed an enrichment in cells that stained positive for VGluT1. Na^+,K^+-ATPase α3 appeared to be highly expressed in both pre- and postsynaptic membranes and showed a particularly high expression in presynaptic membranes of GABAergic cells. These cells generally had long-extending dendrites in the culture. The Na^+,K^+-ATPase α1 isoform on the other hand had a highly distinct postsynaptic expression in cells that had less extended dendritic trees but were also expressed in axons that labeled positive for VGluT1.

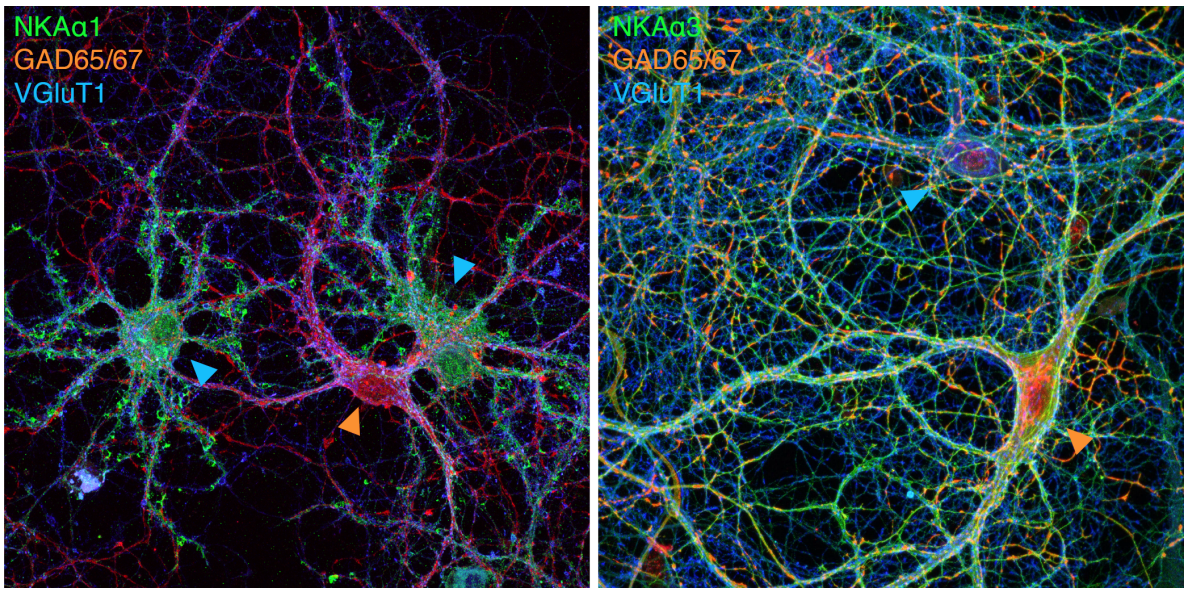

Figure 4.9 Cultured hippocampal neurons. Na^+,K^+-ATPase α1 is predominantly expressed in glutamatergic VGluT1 positive neurons (blue arrow head), Na^+,K^+-ATPase α3 predominantly expressed in GABAergic GAD-65/67 positive neurons (red arrow head).

5 GENERAL DISCUSSION

5.1 NORBIN AND P11 REGULATION OF MGLUR5 ACTIVITY

We have shown that the two cytosolic proteins Norbin and p11 interact with the C-terminus of mGluR5. Both Norbin and p11 increase cell surface expression of the receptor and positively regulate mGluR5 mediated Ca^{2+} oscillations. Both Norbin and p11 thus facilitate mGluR5 function. Genetic deletion of either of these interacting molecules results in behavioral deficits that correspond to phenotypes seen in mGluR5 KO mice. These impairments are at least in part caused by their interaction with mGluR5 seeing how the p11 and Norbin KO mice show deficits in the response to the mGluR5 specific antagonist MPEP. Norbin KO mice show increased MK-801 induced locomotor activity, which MPEP fails to augment further. The antidepressant effect of MPEP is abolished in both constitutive p11 knockout mice and mice with a specific deletion in GABAergic neurons.

The temporal patterning of Ca^{2+} oscillations generated by mGluR5-activation is largely independent of agonist concentration and correlates to the number of receptors expressed at the cell surface (Nash et al., 2002). In our experiments ectopic expression of mGluR5 activation in HEK293 cells generated Ca^{2+} oscillations that were highly heterogeneous. Some cells responded with a single peak while many cells responded with persistent oscillations. This likely reflects, at least in part, different levels of mGluR5 cell surface expression. The Ca^{2+} oscillations generally faded in both amplitude and frequency until they finally ceased suggesting that the receptor was desensitized, possibly via internalization. Norbin prolonged, but did not change the frequency of the oscillations. In contrast, p11 appeared to modulate the frequency, although mildly in the case of overexpression. Since the oscillations generally faded we chose to quantify the frequency at the beginning of every experiment, during 5 or 10 min after agonist application as this should indicate the abundance of receptors present at the cell surface before massive desensitization.

It remains unclear as to how Norbin and p11 facilitate mGluR5 cell surface expression. One potential interpretation of the Ca^{2+} findings is that Norbin, which prolonged the calcium oscillations, competes with factors that regulate agonist triggered internalization, such as PKC activity. Calmodulin, which partially share mGluR5 binding sites with Norbin have been shown to regulate receptor cell surface expression by interfering with PKC triggered desensitization (Lee et al., 2008). Notably the p11 binding-deficient mGluR5 mutant displayed oscillations that had a lower frequency than the wild-type mGluR5. This could in addition be due a disruption in one of the mGluR5 PKC phosphorylation sites which regulates the oscillatory response of the receptor (Kawabata et al., 1996; Kim et al., 2005). Optimally the Ca^{2+} experiments from these two studies should be repeated under identical conditions to establish whether Norbin and p11 influence the Ca^{2+} by different means, and preferentially in neurons from WT and p11 or Norbin knockout animals. One of the major differences between experiments was the use of stably transfected and transiently transfected cells i.e. the baseline levels of mGluR5 cell surface expression were likely somewhat different between the studies. Nevertheless, based on the observations made in the Ca^{2+} experiments one might speculate that p11 could facilitate mGluR5 insertion in the plasma membrane while Norbin might suppress mGluR5 endocytosis, as different means of

increasing receptor availability. Whether the mGluR5 mediated Ca^{2+} oscillations that can be observed during prolonged pharmacological stimulation would actually occur under synaptic glutamate release and bear any physiological relevance in neurons remains to be determined. However, they have been observed in developing cortical neurons under endogenous glutamate release (Flint et al., 1999). The frequency of such oscillations has been demonstrated to define various physiological processes by controlling the activation of distinct Ca^{2+} binding proteins and transcription factors (Dolmetsch et al., 1998; De Koninck & Schulman, 1998; Spitzer, 2012; Smedler & Uhlen, 2014).

Further investigations on how Norbin and p11 control trafficking and downstream signaling are needed to fully understand their role in mGluR5 dependent synaptic activity. Norbin and p11 could both simply facilitate the activity of mGluR5 by maintaining the receptor at the cell surface. However, they could also modulate neuronal function by tuning mGluR5-dependent downstream processes. Protein synthesis is a fundamental feature of long-lasting changes in synaptic strength (Sutton & Schuman, 2006) and many of the lasting changes that are triggered by mGluR5 activation is dependent on mRNA translation. Chemical activation of mGluR5 or paired-pulse low-frequency stimulation triggers mGluR5-mediated LTD in CA1 pyramidal neurons which is blocked by protein synthesis inhibitors cycloheximide and anisomycin (Huber et al., 2000). Changes in spine morphology that can be induced by a Group1 mGluR agonist requires both Ca^{2+} mobilization and protein synthesis (Vanderklish & Edelman, 2002).

The endoplasmic reticulum is a continuous network that can extend from the dendrite into the spine to supply a local Ca^{2+} store at the synapse (Berridge, 1998). In CA1 pyramidal neurons, ER is mainly found in a subpopulation of large spines which contain strong synapses (Holbro et al., 2009). Local low-frequency two-photon glutamate uncaging at the ER-containing synapses triggers a confined depression in synaptic strength that is dependent on group1 mGluR and IP_3R (Holbro et al., 2009). ER lacking weaker spines do not undergo this mGluR dependent depression, suggesting that group 1 mGluR-mediated Ca^{2+} signaling in mature dendritic spines counterbalance the synapses from growing too strong. This indicates that mGluR5-mediated Ca^{2+} signaling specifically can activate downstream processes which balance excitatory activity with a confinement to individual spines. Interestingly, the protein synthesis machinery can be located at the base of the spine, and dendrites are able to serve synapses with newly synthesized proteins in a local regime (Bodian, 1965; Sutton & Schuman, 2006; Brismar et al., 2014). mGluR5-mediated LTD triggers AMPA receptor endocytosis which is dependent on rapid translation of Activity-regulated cytoskeleton-associated protein (Arc) (Waung et al., 2008). An interesting future topic would be to study whether Norbin and p11 could regulate the protein synthesis in single dendritic spines by facilitating or tuning mGluR5 activity locally. Such a study would be possible by using Multi-isotope imaging mass spectrometry (MIMS) as we have established in collaboration with NRIMS at Harvard (Brismar et al., 2014).

The mGluR5 C-terminus is a hot spot for receptor-protein interaction. In addition to Norbin and p11, there are several known regulators such as Homer, Calmodulin, Spinophilin, Tamalin and PKC. This alone suggest that mGluR5 is subject to a great degree of modulation by synaptically localized partners, of which some even overlap in binding sites and likely compete in their regulation. To what extent they are expressed in the same brain regions and

cell types remains to be determined. The differences in behavioral phenotypes observed in the Norbin KO and p11 KO mice could, in part at least, be caused by the cell-type specific expression patterns. In hippocampus the highest expression of Norbin is seen in glutamatergic pyramidal and granule cells while p11 is enriched in interneurons. They also appear to have diverging distributions in cortical layers. The cell-type specificity of Norbin and p11 expression in the brain could give some clues in the role of mGluR5 in depression and schizophrenia.

Data from pilot experiments indicate that overexpression of Norbin decreases the mobility of mGluR5 in dendritic spines while increasing the mGluR5-mediated dendrite and spine outgrowth in hippocampal neurons. This suggests that Norbin might facilitate clustering of mGluR5 in the dendritic spine to promote mGluR5-induced spine growth. One possible explanation by which Norbin could modulate mGluR5 function is via its coupling to actin. Considering the important role of actin in the reshaping of spine morphology and in the control of synaptic strength, the study of a possible direct interplay between mGluR5, Norbin and actin may provide some insight. An interesting experiment would be to disrupt the association between Norbin and actin and study the localization, mobility and functional effects on downstream signaling of mGluR5.

5.2 NMDA RECEPTOR-NA^+,K^+-ATPASE INTERACTION

Study IV describes an anatomical and functional coupling between the NMDA receptor and the Na^+,K^+-ATPase which may have implications for protection against excitotoxicity as well as for the regulation of synaptic strength.

Activation of the NMDA receptor triggers a robust Ca^{2+} influx. Nanomolar concentrations of ouabain, which did not disrupt the resting membrane potential, rapidly attenuated the NMDA-triggered Ca^{2+} influx. The effect of ouabain was both immediate and reversible and specific for the NMDA receptor among the Ca^{2+} mobilizing glutamate receptors. Ca^{2+} studies in HEK293 cells confirmed the effect of ouabain on NMDAR triggered Ca^{2+} influx and provided additional information on the relative roles of NR2A and NR2B as well as Na^+,K^+-ATPase α1 and α3. The experiments performed in the HEK293 cells indicate that the down-regulating effect of ouabain is specific for Na^+,K^+-ATPase α3, and possibly for NMDA receptors containing the NR2A subunit. The NMDA-triggered Ca^{2+} response was larger in cells transfected with Na^+,K^+-ATPase α3 than cells only expressing the endogenous α1 which would suggest that α3 positively modulates NMDA receptor function, in the absence of ouabain.

The Na^+,K^+-ATPase cycles between its Na^+-binding state and its K^+-binding state. Ouabain locks the α subunit in the K^+ binding conformation (Nyblom et al. 2013; Morth et al., 2007; Ogawa et al., 2009) which may influence the binding of the Na^+,K^+-ATPase to the NMDA receptor. The effect of ouabain is likely not an effect of ouabain binding to the NMDA receptor itself considering that the effect appear to be absent in HEK293 cells only expressing Na^+,K^+-ATPase α1.

One of our initial ideas on how ouabain could regulate NMDAR activity was via the Src family of tyrosine kinases (SFK). SFK has been extensively studied as a major source of post-translational modifications that modify NMDAR activity (Salter & Kalia, 2004).

Na^+,K^+-ATPase has been proposed to form a functional complex with Src which can be regulated by ouabain (Tian et al., 2006). Here, SFK turned out not to have an impact on the ouabain effect. Ouabain, in fact, caused a slight increase in SFK-mediated NR2B phosphorylation which could augment NMDA receptor activity.

The dSTORM data show that the Na^+,K^+-ATPase and the NMDA receptor can be close enough for a direct interaction to occur. Every other NR2A and NR2B subunit had a Na^+,K^+-ATPase α3 isoform closer than 50 nm. Although our GST pull-down experiments needs to be extended, our preliminary results suggest that the Na^+,K^+-ATPase α isoform contain regions that can interact physically with the NMDA receptor. To confirm that this direct interaction can results in an allosteric effect via ouabain, additional experiments are needed.

Our observations that the Na^+,K^+-ATPase α isoforms are differentially expressed in hippocampal glutamatergic and GABAergic neurons, and that the effect of ouabain appears to be restricted to HEK293 cells overexpressing the α3, implicate that an endogenous regulation of the NMDA receptor would occur predominantly in GABAergic neurons. We are expanding on these observations and are at present studying the distribution of the different α subunits in the adult rodent brain. Our preliminary findings suggest that the α subunits are differentially expressed throughout the brain.

Ca^{2+} influx via the NMDA receptor is essential in modulating synaptic activity (Lüsher & Malenka, 2012). Overactivation of NMDA receptors however can trigger excitotoxic events via Ca^{2+} overload and mitochondria dependent cell death (Lipton & Rosenberg, 1994; Simon et al., 1984; Lemasters et al., 2009). Low-doses of ouabain have been shown to protect from damages associated with persistent NMDA receptor mediated Ca^{2+} influx in mouse models of ischemic stroke and traumatic brain injury (Wang et al., 2006; Dvela-Lewitt et al., 2014). Our data might provide insights on the mechanism of the neuroprotective effects of ouabain seen in these models.

Important in future investigations will be to determine the nature of the functional interaction between the Na^+,K^+-ATPase and the NMDA receptor. Förster resonance energy transfer (FRET) experiments should help to address the question on whether ouabain could dynamically influence a physical coupling between the Na^+,K^+-ATPase and the NMDA receptor via an allosteric mechanism. As the Ca^{2+} flux as we observed, is a rather indirect measure of the activity of the NMDA receptor and inherently mixed by multiple components that contribute to Ca^{2+} homeostasis, an experiment in which the currents through the NMDA receptor directly is measured could give further support in the specificity of the effect. Moreover, the long-term physiological effects of ouabain on NMDA receptor activity, such as an effect on NMDA associated LTP and in NMDA receptor related excitotoxic models could help in our understanding of this novel functional relationship.

5.3 SUPER-RESOLUTION MICROSCOPY

In two of the studies presented here, super-resolution microscopy was implemented to investigate the localization of proteins in postsynaptic membranes and structures. The improvement in resolution allowed us to explore the distribution of fluorescent molecules on a subsynaptic scale.

Using SIM with a resolution of ~100 nm in the lateral directions, we could dissect the localization of synaptic proteins in relation to structural elements in the dendritic spine. Some of these proteins, such as Norbin and mGluR5, were expected to give a high degree of colocalization. More interestingly perhaps was the fact that Norbin and Actin had an even higher degree of colocalization, and that Norbin showed a relatively weak correlation with PSD-95. Together these results suggest that Norbin plays a different role than other mGluR5 scaffolding proteins located in the spine head. We were also able to confirm these observations using biochemical methods. In addition, we compared the results of SIM imaging of exogenously expressed Norbin and PSD-95 with STED imaging of endogenously labeled proteins and found them to give equivalent results in terms of overall localization. While SIM relies on post-acquisition analysis, STED provides a super-resolved raw image without any requirement of further analysis.

In study IV, using dSTORM and PALM, we set out to estimate the distances between labeled proteins on the nanoscale. An important aspect of different labeling techniques when studying the sample with single-molecule localization techniques is that the proteins per se are not studied but the positions of the linked fluorescent molecules. In the study of protein localization in antibody-based approaches this is a particularly important point since the precision can be measured on the scale of the size of the antibody complexes themselves. Therefore, the development of novel labeling strategies and production of smaller labeling probes such as nanobodies is of great importance. Nevertheless, the antibodies are likely to be randomly displaced in relation to the labeled protein in the sample. The measured position of the fluorescent dye molecule will consequently, on average, will be closer to the labeled protein than the size of the primary-secondary antibody complexes under 2D imaging conditions as employed here.

The nearest neighbor approach as we implemented it here will not find unique nearest neighbors or give information about the relative abundance of molecules in e.g. a cluster of proteins. It simply finds the nearest neighbor in relation to all localized molecules. The nearest neighbor in combination with cluster analysis to quantify how many molecules are in within macromolecular complexes, such as would be expected in synapses, should likely give a better understanding of the functional organization of proteins.

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